Vitamin E and egg production in heat stressed laying hens

Sandy BOLLENGIER-LEE

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Declaration of originality

I declare that I am the author of this thesis and, except where indicated, am solely responsible for the experimental work described within.

Signed,

Sandy Bollengier-Lee

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Abstract

Egg production has long been known to be depressed in laying hens exposed to chronic periods of heat. Such conditions, experienced by layers during the summer months in Europe and all the year round in tropical countries, result in large economical losses to the poultry industry. The objective of the work described in this thesis was, firstly, to confirm that vitamin E has a beneficial effect on egg production in heat stressed laying hens and, secondly, to investigate the molecular basis of its effect.

During exposure to a chronic period of heat (4 weeks at 32°C), egg production was improved in birds fed vitamin E-enriched diets. Distribution of a diet containing 250 mg vitamin E/kg diet before (4 weeks), during and after (8 weeks) the period of stress increased egg production by 12% ($P<0.02$) during and by 13% ($P<0.02$) in the four weeks following the stress compared to control birds (fed 10 mg vitamin E/kg diet).

Egg formation involves the mutually dependent activities of the ovary (uptake of yolk precursors and synthesis of oestrogens) and of the liver (oestrogen induced-synthesis of yolk precursors). Neither heat stress nor vitamin E were found to have direct effects on the *in vivo* uptake of the main yolk precursor protein, vitellogenin, by oocytes or on the circulating concentration of 17β-oestradiol. Although the amount of vitellogenin messenger RNA in hepatocytes and vitellogenin protein in the circulation were significantly depressed during heat stress in non-supplemented birds, the concentration of vitellogenin protein in hepatocytes was dramatically increased in these birds (+63%, $P<0.01$) compared to pre-stress values. In vitamin E-supplemented birds, this accumulation of vitellogenin protein in hepatocytes did not occur and the circulating vitellogenin concentration was higher than in non-supplemented birds.

It is therefore proposed that, during heat stress, vitamin E improved the export of vitellogenin from the liver into the circulation. This effect is probably related to the known antioxidant properties of vitamin E and to its consequent protective effect on the integrity of organelle and plasma membranes which is required for proper export of proteins through the secretory pathway.

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Chapter 1

Introduction

The objectives of the experiments documented in this thesis were to investigate whether dietary vitamin E supplementation has a beneficial effect on egg production in laying hens exposed to chronic heat stress and to try to establish the mechanism(s) involved. A series of nutritional trials (experiments 1 and 2) carried out on large numbers of birds under field conditions established the optimal regimen of vitamin E supplementation that would lead to improved egg production in heat-stressed laying hens. The remaining experiments (experiments 3 to 9) were designed to understand the mechanism(s) by which vitamin E improved egg production during heat stress. A smaller number of birds were used in these experiments and they received the optimum regimen of vitamin E established above. This work utilised a wide range of biological techniques including biochemical and molecular biological methodologies as well as electron microscopy.

The large scale nutritional trials were carried out at the experimental farm of Rhone-Poulenc Animal Nutrition in France whilst biochemical and other studies were performed at the Roslin Institute in Scotland. The order in which the experiments are reported in this thesis reflects the chronological progress of the work.

Current knowledge and literature on the process of egg production, the influence of heat stress in laying hens and the effect of vitamin E on heat-stressed poultry are reviewed in **Chapter 2.** The materials and methods common to all the experiments are then presented in **Chapter 3.** The economical justification for vitamin E supplementation is documented in **Chapter 4** in which both the effect of vitamin E on egg production and the optimal regime (dose and duration) of supplementation were investigated in large scale experiments.

Once the beneficial effect of vitamin E was demonstrated and the optimal dose determined, the mechanism of action of vitamin E was investigated by studying the effects of vitamin E on the

two main organs involved in egg production, the ovary and the liver. The ovary, composed of oocytes, is the site of uptake of yolk precursors in yolks and a major site of production of reproductive hormones. Therefore, the effect of vitamin E on this organ was studied in the next five experiments. To study the influence of vitamin E on the uptake of the main yolk protein precursor, vitellogenin, by oocytes *in vivo*, a technique whereby vitellogenin was radiolabelled, injected into birds and detected in the oocytes was developed and validated is described in **Chapter 5**. This technique was then used as described in **Chapter 6** to quantify and compare the uptake of vitellogenin by oocytes in normal and heat stressed birds and in non-supplemented and vitamin E-supplemented birds. As heat stress and vitamin E could also influence egg production by influencing the concentration of reproductive hormones in the circulation, the plasma concentration of a major reproductive hormone, 17β-oestradiol, responsible for the stimulation of the gene encoding for the vitellogenin protein was determined in **Chapter 7**.

As vitellogenin is synthesised in the liver, the effect of heat stress and vitamin E on the activity of the vitellogenin gene and on the amount of vitellogenin protein were studied in hepatocytes in **Chapter 8**. The subcellular localisation of the vitellogenin protein was then investigated within hepatocytes of normal and heat stressed birds and of non-supplemented and vitamin E-supplemented birds in **Chapter 9**.

Finally, in **Chapter 10**, the main observations and conclusions drawn in each experiment are discussed and a mechanism by which vitamin E improves egg production during heat stress is proposed.

Chapter 2

Literature review

2.1. Egg production

In no other sector of animal production has the utilisation of scientific knowledge had such an impact on economic efficiency than in poultry production. This is reflected by the tremendous increase in the performance of laying hens over the last half century as seen in Table 1, which shows the evolution of egg production in the United States since 1940. In the most widely used strain of brown egg layers in the world, Isa Brown, egg production has increased from 239 to 301 eggs per hen per year and the age at which a production of 0.5 eggs per hen per day is reached has been decreased from 26 to 21 weeks (I.S.A., 1993a).

Table 1. Improvement in egg production of laying hens. From U.S. Department of Agriculture (1990).

Year	Eggs/hen annually*	
1940	134	(100)
1950	174	(130)
1960	209	(156)
1970	218	(163)
1980	242	(181)
1990	250	(187)

* Values in parentheses are percentages relative to 1940

The increase in performance of laying hens has resulted in a considerable rise in egg production in the world, particularly in the developing regions (Table 2) (Daghir, 1995). As these regions are also characterised by a low but rising per capita egg consumption, 73.5 eggs consumed per capita in 1990 in Africa, South America and Asia, versus a plateau value of 222.9 in the rest of the world, the rise of the egg industry in developing regions which include most of the hot regions of the world will probably continue in the future.

Table 2. World production of eggs in million metric tons.
From FAO (1990) and Poultry International (1997).

	Egg production (per year)		
Continent	1980*	1990*	1996*
Africa	0.92	1.42	1.70
South America	1.56	2.31	2.60
Asia	7.58	14.27	24.51**
North and central America	5.42	5.79	6.45
Europe	7.22	7.17	9.26**
Oceania	0.26	0.25	0.20
Former USSR	3.76	4.54	/**
World	**26.74**	**35.76**	**44.71**

* : Data for 1980 and 1990 were obtained from FAO (1990) and data for 1996 were obtained from Poultry International (1997)

** : In 1996, egg production of Former USSR was included with those of Asia and Europe

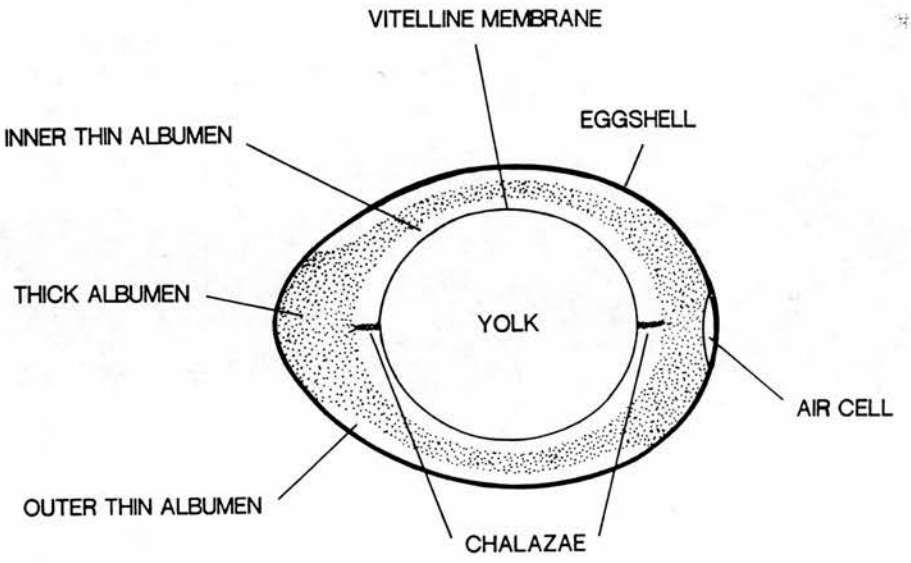
2.1.1. Egg composition

An egg is composed of three main fractions : yolk, albumen and shell (including shell membranes), which account for, respectively, 32%, 56% and 12% of the weight of the total egg. The composition of these fractions is presented in Table 3. An egg of 60 g yields 90 kcal of energy and contains as much lipid as protein, 6.5 g of each. These components are exclusively (lipids and energy) or largely (proteins) located in the edible part of the egg. Within the edible part, all the lipids, 48% of the proteins and 71% of the energy are located in the yolk. The physical arrangement of the three fractions of the egg is shown in Figure 1.

Table 3. Content of lipid, protein, carbohydrate, ash, water and energy in yolk, albumen and shell in an egg of 58 g. From Romanoff and Romanoff (1949) and Burley and Vadehra (1989).

	Yolk	Albumen	Shell and shell membranes
Water (g)	9.1 - 9.3	28.4 - 29.0	-
Solids (g)	9.1 - 9.4	3.5 - 4.0	6.8
Protein (g)	3.0 - 3.1	3.1 - 3.5	0.45
Lipid (g)	5.7 - 6.3	trace	trace
Carbohydrate (g)	0.11 - 0.13	0.3	trace
Ash (g)	0.3	0.22 - 0.23	6.4
Energy (kcal)	65 - 70	24- 30	-

Figure 1. Physical arrangement of the yolk, albumen and shell within the egg. From Etches (1996).



2.1.1.1. Yolk composition

The yolk, which is surrounded by four concentric layers that together make up the yolk membrane, is mainly composed of yellow yolk deposited in concentric layers around the core of the yolk. This core is composed of "white yolk" which contains less protein and lipid and more water than the yellow yolk (Spohn and Riddle, 1916). The yellow yolk is made of tightly packed yolk spheres (approximately 140 μm in diameter) containing proteins and lipids in subdroplets, particles, lamellar bodies and a finely dispersed aqueous phase.

The subdroplets (100 to 2,500 nm in diameter) contain about 23% of the yolk protein in the form of two phosvitins and two lipovitellins which, as a result of their high number of serine residues, bind a variety of metal ions, such as phosphorus, zinc, calcium, magnesium, copper, iodine, manganese. The phosvitins account for about 80% of the protein-bound phosphorus within the yolk and 90% of the protein-bound iron and have molecular masses of 28 and 34 kDa (Clark, 1973). The α- and β-lipovitellins, which account for 90% of the protein-bound zinc, differ in their phosphorus content and exist as dimers which can be dissociated into identical monomers with molecular masses of about 200 kDa.

The particles contain about 65% of the yolk solids and 95% of the yolk lipids. These structures are about 27 to 35 nm in diameter and contain the very low-density lipoproteins

(VLDLs). The VLDLs are large macromolecules which have molecular masses between 3,000 and 10,000 kDa and contain 12% protein and 88% lipid. The protein component of each VLDL particle contains one molecule of apolipoprotein-B (apo-B) and as many as 25 molecules of apo-VLDL-II and these molecules facilitate the entry of VLDL into the yolk. Of the lipid contained in the particles, 70-75% is triglyceride, 20-25% is phospholipid and 4% is cholesterol. Although greatly influenced by the composition of the diet (Sell *et al.*, 1968), the fatty acid composition of the triglycerides and of the two most common phospholipids, lecithin and cephalin, reveals that palmitic acid and oleic acid are the most common components of VLDL particles and, hence, of yolk lipids (Table 4). In addition to their location in particles, VLDLs probably also exist in a membrane-like form within the yolk spheres as lamellar bodies.

Table 4. Partial fatty acid composition of triglycerides and phospholipids in VLDL complexes in egg yolk. Modified from Burley and Vadehra (1989).

Class of lipid	Fatty acids (moles per 100 moles)						
	Palmitic (C16:0)	Palmito-leic (C16:1)	Steraic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)	Linole-nic (C18:3)	Arachi-donic (C20:4)
Triglyceride	22 - 28	5 - 7	5 - 7	44 - 46	12 - 15	1	1
Lecithin	32 - 44	1 - 3	12 - 17	27 - 30	5 - 16	1	2 - 4
Cephalin	16 - 33	2 - 35	17 - 32	7 - 28	2 - 11	10	3 - 16

The aqueous phase contains approximately 10% of the yolk solids, most of which are proteins. The major proteins are albumin, α_2-glycoprotein and the immunoglobulin subclass IgG. The minor proteins include a number of carrier molecules for biotin and other vitamins, which explains the fact that most of the vitamins of the egg are located in the yolk.

2.1.1.2. Albumen composition

Although Lush (1961) distinguished 19 major components of albumen, seven proteins account for more than 90% of its dry matter. Among these proteins are ovalbumin which makes up 54% of the albumen, ovotransferrin, ovomucoid, ovomucin and lysozyme which together make up another 30% of the albumen (Powrie and Nakai, 1986). As albumen contains as much protein as does yolk and 75% of the water reserves of an egg, it constitutes an important source of nitrogen and a major source of water for the developing embryo. Albumen also contains

traces of lipid, a few vitamins (78% of the niacin and 61% of the riboflavin is located in the albumen) and approximately a third of the mineral content of the egg.

2.1.1.3. Shell composition

The shell is composed of several layers that function together as a single unit of about 350 μm width to contain the egg contents and provide a barrier to the entry of micro-organisms into the egg. On its internal side (in contact with the albumen), the shell is composed of two membranes, the inner and outer shell membranes, which are in intimate contact except in the region of the air cell. On the outer shell membrane lies a layer of hexagonal-rhombohedral crystals of calcium carbonate. These calcite crystals, which are themselves covered by a glycoprotein cuticle, constitute the bulk of the shell and impart strength to it.

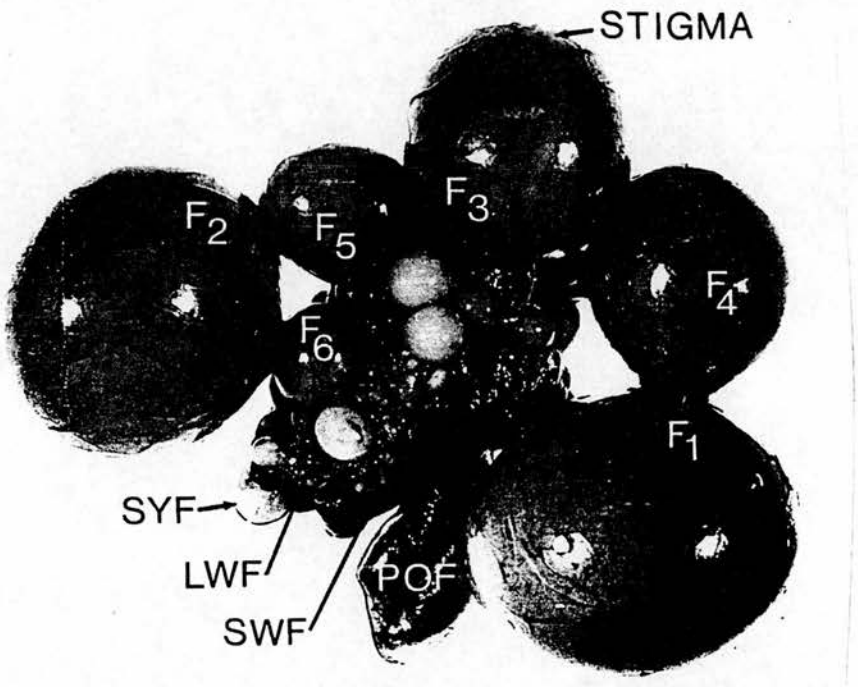
2.1.2. Egg formation

In the mature laying hen, an egg is laid approximately every day. However, the process by which an egg is formed takes much longer than a day. Yolk formation takes place in the ovary (Figure 2), which is composed of a highly vascularised tissue (or medulla) in its core and of a cortex where the follicles are embodied. Albumen and shell formation occurs in the reproductive tract of the female bird (Figure 3).

2.1.2.1. Yolk formation

By the time of hatching, the ovary of a bird contains its full complement of oocytes which are in growth arrest. This population constitutes the stock from which follicles are drawn during the reproductive life of the bird. They have diameters of 0.06 to 0.08 mm and are each composed of an oocyte surrounded by cells of mesodermal origin that differentiate into a layer of granulosa cells. The granulosa cells are surrounded by thecal tissue and separated from it by a basement membrane. The theca is a particularly important tissue as it is highly vascularised and assures a variety of functions within the ovary. It maintains the follicular hierarchy, i.e. the harmonious development of the follicles, the supply of yolk precursors to the follicles, the production of steroids, and the expulsion of the ovum at the time of ovulation. The latter occurs in a particular region located at the apex of the follicle, designated the stigma (Figure 2), where the vascularisation network and connective tissue are absent and which ruptures during ovulation.

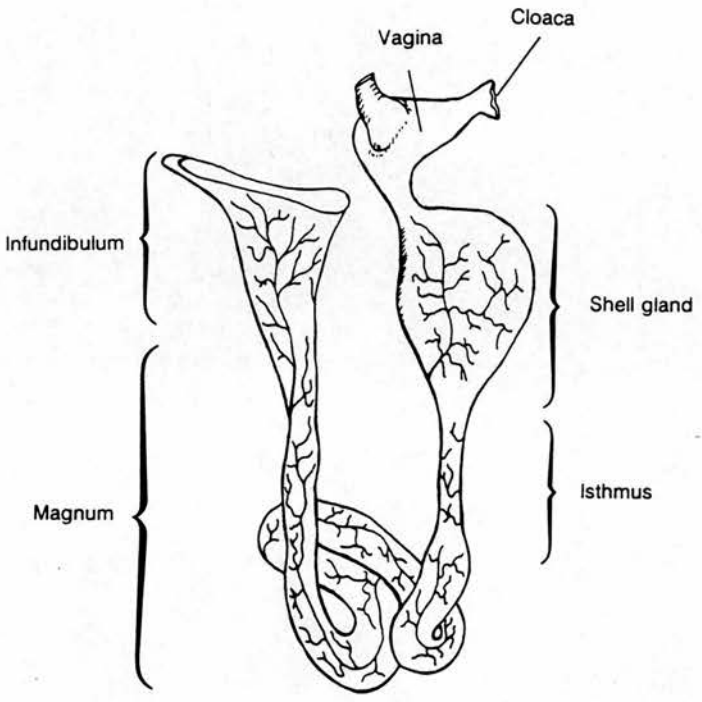
Figure 2. Representation of the ovary containing a hierarchy of yellow yolky follicles identified as the F1, F2, F3, F4, F5 and F6, and thousand smaller follicles from which the large yolky follicles are recruited. From Etches (1993).



The small follicles are classified according to their diameters as small yellow follicles (SYF, 5 to 10 mm), large white follicles (LWF, 2 to 4 mm) and small white follicles (SWF, <1 mm). The stigma, an avascular area of the F_1 follicular wall, ruptures to release the ovum. The ruptured or post-ovulatory follicle (POF) is easily identified for at least 48 h following ovulation and then regresses during the next few days.

On initiation of growth, the oocyte undergoes a succession of changes in preparation for yolk formation (Bellairs, 1967; Chalana and Guraya, 1979). During the initial stage of development of the yolk (0.07 to 2.5 mm), complex macrobodies are formed in the superficial cytoplasm over a period of 4 to 5 months (Paulson and Rosenberg, 1972). These macrobodies result from the phagocytosis of specialised fragments of the surrounding granulosa cells known as lining bodies, transosomes or unique organelles (Bellairs, 1967). In the deeper cytoplasm, similar bodies arise from clusters of organelles produced by the oocyte (Bruce, 1977). These organelles are important for the synthesis of yolk proteins and membranes (Schjeide *et al.*, 1975). The macrobodies are later transformed into primordial yolk spheres which possess single or several dense subdroplets embedded in a less dense matrix of granular material.

Figure 3. Anatomy of the reproductive tract of the hen, illustrating the infundibulum, magnum, isthmus, shell gland and vagina. From Etches (1996).



In the first vitellogenic phase (2.5 to 4 mm), which lasts for 6 to 8 weeks, definitive yolk spheres or white yolk appear in oocytes and form the bulk of the cell, which in turn forms the bulk of the ovarian follicle. It is followed by a 24 h transitory phase (4 to 8 mm), in which phagocytosis ceases, phosvitin and immunoglobulins taken up by pinocytosis increase to form a yellowish yolk (Cutting and Roth, 1973). The process gathers momentum in the main vitellogenic phase and results in the production of a deeper yellow yolk which reaches a diameter of 37 mm. During the main vitellogenic phase, which occurs over a period of ten days preceding ovulation, VLDL and vitellogenin are taken up by the oocyte resulting in the deposition of yolk in radial increments of about 2 mm per day in follicles of 7 g (Oribe *et al.*, 1975) and continues in most oocytes until 2 to 3 h before ovulation (Gilbert, 1971). The mechanisms by which VLDL particles enter and contribute to the formation of the yolk are described in detail in section 2.1.5.4.

2.1.2.2. Ovulation

The release of the ovum from the follicle at ovulation occurs when the stigma ruptures, leaving the post-ovulatory follicle attached to the ovary. Expulsion of the ovum is facilitated by the physical structure of the follicle and the secretory capacity of the fibroblasts (collagenase and

other proteases) in the stigma and is assisted by the contractions of myofibrils in the follicular wall. The ovum is shed from the ovary surrounded only by the vitelline membrane and the perivitelline layer that was deposited by the granulosa cells during follicular growth. The post-ovulatory follicle, which begins to degenerate after ovulation, retains its capacity for synthesis of steroids and prostaglandin for at least 24 h after ovulation. The post-ovulatory follicle or its granulosa cells also produce the prostaglandins which are involved in the oviposition, e.g. the expulsion of the completed egg from the vagina.

2.1.2.3. Albumen formation

After ovulation, the yolk is captured in the infundibulum (Figure 3) where the outer layer of the vitellin membrane is secreted over a period of 20 minutes. This membrane prevents the transfer of water from the albumen into the yolk. The yolk then moves to the magnum (Figure 3), where the albumen is secreted. This secretion is particularly active just after a yolk has transited through the magnum and is regulated by a complex hormonal control (section 2.5.2). The transit of the yolk through the magnum takes 3.30 h and provokes the release of the proteins accumulated in the magnum cells by physical stimulation. When the egg leaves the magnum, the albumen contains 50% its final water content, and most of its final sodium, calcium and magnesium content.

2.1.2.4. Shell membrane and shell secretion

As soon as the egg arrives in the isthmus (Figure 3), the albumen is covered with collagenous fibres which intermesh to constitute the inner and outer egg shell membranes. During this 60 minute period, the deposition of the fibres is continuous as the egg progresses in the isthmus. The secreted material is produced by tubular glands and swells on contact with water to form a dense fibrous layer. In the terminal portion of the isthmus, the organic matrix of the shell (mamilliary layer) is secreted. The membrane fibres are incorporated into the shell in these mamilliary cores which serve as nucleation sites for the growth of hexagonal-rhombohedral crystals of calcium carbonate which constitute the main palisade layer of the shell.

Depending on the length of the light period (or photoperiod), an egg spends 18 to 22 h in the shell gland (Figure 3). During the first phase, which last 6 h and which is called "plumping", the amount of water in the albumen doubles resulting in the tension of the shell membranes.

The secretion of sodium, potassium and bicarbonate is also resumed, but decreases 12 h after ovulation. Part of the sodium is then reabsorbed. Before the albumen is fully hydrated (10 h after ovulation), the deposition of calcium carbonate crystals starts. During calcification, which lasts 12 h, between 0.30 to 0.35 g of shell is deposited per hour to reach a maximum thickness of approximately 350 µm. The cessation of calcification occurs 2 to 4 h before oviposition and is caused by an steroid controlled-enrichment of the uterine liquid in phosphates. The superficial coloration of the shell is due to pigments called ooporphyrines and occurs at the end of the calcification. The cuticle is secreted in the last 22 h after ovulation.

2.1.3. Formation of egg precursors

2.1.3.1. Yolk synthesis

Studies by Flickinger and Rounds (1956) on the incorporation of ^{32}P into liver, blood, and yolk proteins suggested that the liver is the site of synthesis of yolk proteins. This was confirmed by Clemens *et al.* (1975) who demonstrated that liver preparations *in vitro* are capable of synthesising and secreting egg-yolk proteins. However, some of these proteins are not directly synthesised in the form which is found in the yolk. This is the case of the phosvitins and lipovitellins which have been shown to result from a single plasma precursor, vitellogenin, synthesised in the liver (Bergink and Wallace, 1974). Vitellogenin, which is a dimer with molecular weight of 480 kDa, is transported to the ovary through the vascular system and deposited in the growing oocytes by receptor-mediated endocytosis (section 2.1.5.4.). As vitellogenin enters the ovum, it is cleaved into two phosvitin and one lipovitellin units (Gordon *et al.*, 1977). The plasma concentrations of vitellogenin and apo-VLDL-II, which are very low in the plasma of immature hens or roosters, increase dramatically in response to oestrogen, and the similarity of the kinetics of their induction suggests their synthesis is subject to co-ordinated regulation (Muniyappa and Adiga, 1980). The hepatic synthesis of vitellogenin and apo-VLDL-II appears to be completely dependent on oestrogen, since neither protein is normally synthesised in rooster liver (Deeley *et al.*, 1977).

Oestrogen is synthesised by the developing follicles in the ovary (section 2.1.4.4.) and, after entering the hepatocyte by simple diffusion, binds to a specific receptor protein and activates the transcription of the vitellogenin gene (section 2.1.4.5.). In rooster liver, vitellogenin mRNA has been reported to begin to accumulate 30 minutes after exogenous oestrogen stimulation, but does not reach its maximal rate until several hours (Deeley *et al.*, 1977) or days (Jost *et al.*,

1978) later. As there is a high correlation between the rate of vitellogenin synthesis and the level of vitellogenin mRNA, there is good evidence that for steroid-hormone-responsive systems (O'Malley and Means, 1974) regulation occurs at the level of mRNA supply rather than at the level of its translation.

Although the overall rate of protein synthesis in the liver is increased by oestrogen treatment, it has been observed that the rate of synthesis of certain specific yolk proteins, most notably serum albumin or apo-A-I, the major apolipoprotein of high-density lipoproteins, declines. Thus during oestrogen-stimulated yolk-protein synthesis in roosters, the albumin level in the circulation decreased by up to 50% (Bergink *et al.*, 1974). This decrease was attributed to a decrease in the rate of synthesis of the protein in the liver, which in turn resulted from a decrease in the content of albumin mRNA (Farmer *et al.*, 1978). The explanation seemed to be that the oestrogen-receptor complex is acting as a negative control element for the albumin gene at the same time as it acts as a positive regulator of the vitellogenin gene (Green, 1980).

2.1.3.2. Albumen synthesis

The egg-white proteins are produced in the magnum and more particularly in the tubular glands which produce at least 80% these proteins. They are the source of ovalbumin, lysozyme, ovotransferrin and ovomucoid, but it has not been established if all of the cells within a tubular gland secrete the same protein or a mixture of proteins, and the physiological regulation of the production and deposition of these proteins has not been elucidated. Other cells, such as the epithelial cells lining the lumen of the reproductive tract also produce some proteins and the secretion of avidin and probably ovomucin is restricted to these cells. The production of albumen appears to proceed continuously within the secretory cells located in both the epithelium and the tubular glands and to be stored in granules. The production and release of these granules seem to follow the classical pattern described for many types of cells that secrete proteins (production in the endoplasmic reticulum, packaging by the Golgi apparatus and coalescence into large storage granules). Although the expression of the genes encoding the production of ovalbumin, conalbumin, ovomucoid and lysozyme is induced by a continuous oestrogen treatment (O'Malley *et al.*, 1969), avidin synthesis has been shown to be progesterone dependent (Sauveur, 1988a).

2.1.4. Hormonal regulation of egg formation

Reproduction and egg formation are strictly controlled by the endocrine system and particularly by the reproductive hormones. These hormones, which are produced by a number of organs, i.e. hypothalamo-pituitary axis, adrenal gland and ovary, are strongly influenced by lighting regimes.

2.1.4.1. Photoperiodism, onset of lay and ovulatory cycle

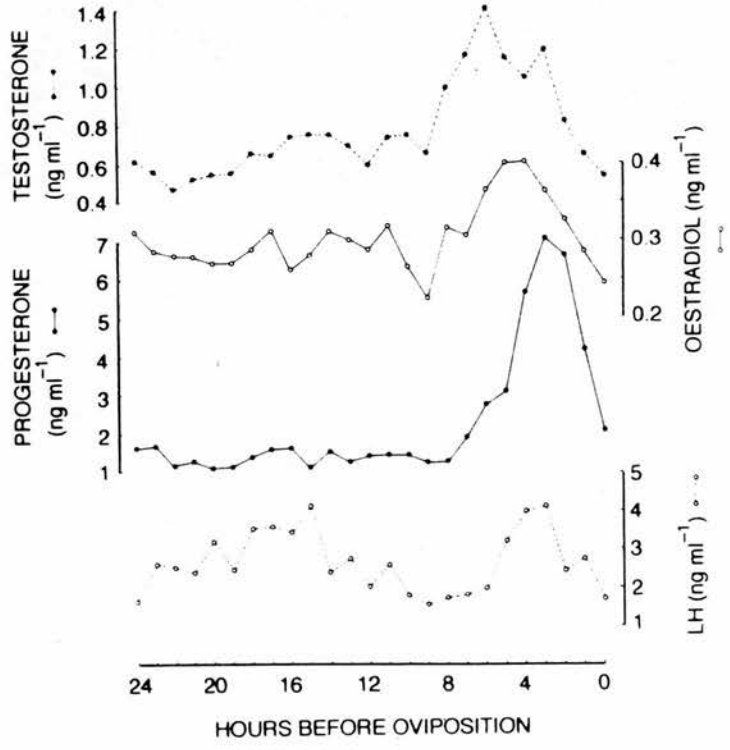
Manipulation of the photoperiod is one of the most powerful management tools available to the poultry producer to influence egg production. The onset of lay can be advanced or retarded, the rate and timing of lay can be influenced and egg size and shell quality can be optimised by providing the appropriate lighting regime. The most common photoschedule provides a single period of illumination, or photophase, of 14 h and a single period of darkness, or scotophase, of 10 h (14L : 10D). Alternatively, some lighting regimes can contain more than one photophase and scotophase which recur at 24 h intervals or provide periods of light and dark that, in combination, may be longer or shorter than 24 h (ahemeral). Although the onset of reproductive activity can be advanced and retarded by the photoschedule, sexual maturation will eventually occur in the absence of a photostimulatory lighting regime. For example, Wilson and Cunningham (1980) found that hens could commence egg production by 24 weeks of age when exposed to 8L : 16D throughout their life but that commencement of egg production could be advanced to about 22 weeks by photostimulation. This process, which results from a direct stimulation of the hypothalamic photoreceptors that transmit their signal to the gonadotrophin releasing hormone (GnRH) secreting neurones of the hypothalamus, causes an increase in the plasma concentrations of luteinising hormone (LH) and follicle-stimulating hormone (FSH).

Once the hen has started to lay, ovulation occurs in sequences that vary in length from two eggs to as many as 360 eggs. The ovulatory cycle of hens, defined as the interval between consecutive ovulations, has been shown to vary from a minimum of 25 to 29 h (Etches, 1996). When a sequence is completed, one or more "pause days" then follow on which no eggs are laid. It was also demonstrated that follicular rupture is restricted to about 8 h of the day, beginning at approximately 6 a.m. and ending at approximately 2 p.m. for hens housed in a room where lights came on at 6 a.m. and went off at 8 p.m. (Fraps, 1955). The 8 h interval during which ovulation spontaneously occurs is referred to as the "open period" which is a

consequence of a circadian rhythm and, therefore, occurs only once during each light-dark cycle under standard photoschedules. As the physiological systems regulating the ability to ovulate in response to the preovulatory LH surge have to be synchronised with the appropriate phase of circadian rhythm, the generation of the preovulatory surge of LH is restricted to an 8 h period of the day.

Studies of the responses of populations of birds to light-dark cycles indicated that the open period for LH release is a circadian rhythm which adapts to ahemeral photoschedules between 21 and 30 h. This was confirmed by Etches *et al.* (1984) who exposed hens to different light-dark cycles and showed that the periodicity of the open period for LH release was equal to the period of the light-dark cycle. The location of the open period within a photoschedule was also shown to be closely linked to the transition from the photophase to the scotophase and, under standard 14L : 10D photoschedules, the mean time of oviposition is approximately 15 h after dusk. Furthermore, as LH stimulates the steroid hormones production by the ovary (section 2.1.4.2.), the preovulatory surge in plasma concentration of LH is accompanied by surges in oestradiol, androgens and progesterone (Figure 4).

Figure 4. Plasma concentrations of luteinising hormone (LH), progesterone, testosterone and oestradiol during the ovulatory cycle of the hen. From Etches (1990).



2.1.4.2. Hormones produced by the hypothalamo-pituitary axis

In addition to its role in the perception of light (section 2.1.4.1.), the hypothalamus plays a central role in the regulation of ovarian function (Sturkie, 1965; Gilbert, 1969). It regulates the secretion of gonadotrophins, FSH and LH, by the anterior pituitary by producing GnRH. The chronic secretion of GnRH into the portal vascular system connecting the hypothalamus to the pituitary gland is under the control of other brain centres and is particularly connected to the photoreceptive centres. Exposure to long days raises the secretion of GnRH, which in turn raises the concentration of gonadotrophins in plasma to levels that support ovarian growth and maintain a hierarchy of ovarian follicles. Although two forms of GnRH have been identified in the chicken hypothalamus, only GnRH-I appears to be involved in the tonic and acute release of gonadotrophin from the anterior pituitary gland and the function of GnRH-II remains enigmatic. In sexually mature laying hens, the GnRH pulses typically recur at intervals of 1 to 3 h and each episode spans 15 to 60 minutes and it is the combination of frequency and amplitude of the pulses that changes the intensity of the message to the anterior pituitary gland.

The gonadotrophins have been named because of their physical and chemical similarities to mammalian hormones, although not all of the functions of the mammalian hormones can also be attributed to their avian homologues. Both LH and FSH are glycoproteins composed of α and β subunits that are held together by non-covalent bonding and it is the β subunit which confers the specificity of their effects on tissues within the gonad. Another gonadotrophin, called prolactin, is release by the anterior pituitary gland and controls hatching behaviour. Although the relative activity of preparations of LH and FSH are not fully known, it is generally accepted that both hormones regulate follicular growth and maintain the large yolk-filled follicles in the hierarchy. Although, under some experimental conditions, FSH may stimulate steroidogenesis and induce ovulation, LH is the most active stimulator of steroid production from the hierarchical follicles and from the non-hierarchical follicles from which they are recruited. The effect of FSH on steroidogenesis may be attributed to LH contaminating the FSH preparations or to unique properties of the hormone that can be attributed to the species of origin of the FSH.

2.1.4.3. Hormones produced by the adrenal gland

The interrelationships between the ovary and the adrenal gland of the hen have been the subject of many investigations. The left adrenal gland is embedded within the ovary and the ovarian

follicles are richly supplied with catecholaminergic nerves. As the endings of these nerves lie in close association with the steroid-producing cells in the thecal tissue, it was suggested that steroidogenesis is neurally regulated. Several studies were carried out to confirm this regulation. Administration of corticosterone, deoxycorticosterone and ACTH to the bird or addition of catecholamines to follicles *in vitro* were shown to induce ovulation in hens. Injections of metyrapone was shown to influence the timing of ovulation and injections of dexamethasone were shown to block ovulation. Follicles of the ovarian hierarchy were shown to grow in an unregulated manner if the ovary was transplanted to the kidney suggesting that the catecholaminergic innervation of the ovary may be essential for the maintenance of the follicular hierarchy. An adequate explanation for these observations has not yet been presented although, taken together, they suggest that ovarian and adrenal function may be connected in an unidentified way (Etches, 1996).

2.1.4.4. Hormones produced by the ovary

Under the control of the gonadotrophic hormones, the ovary cyclically secretes three major classes of steroid hormones, the oestrogens, androgens and progesterone. Steroidogenesis has been studied in detail in the domestic hen (Huang and Nalbandov, 1979; Nakamura *et al.*, 1979).

Although several components of the ovary appear to be able to produce oestrogen, the outer wall of the growing follicles is probably the most important source. At an early stage of follicular development that has not yet been identified, the small ovarian follicles begin to produce oestrogens. Although a wide range of substances has been shown to have oestrogenic activity, such as the phytoestrogens (e.g. flavones, isoflavones and coumestans) in plants, the main oestrogens in birds are represented by 17β-oestradiol, oestrone, oestriol and 16,17-epioestriol. They have been shown to derive from a common precursor, cholesterol, and be synthesised markedly two or three weeks before the onset of lay. In the adult, the oestrogens are synthesised by the second and third largest follicles (Table 5) and the ability to synthesise oestrogens vanishes almost completely the day before the follicle is released from the ovary.

Table 5. **Oestradiol content of ovarian follicles (ng per follicle) in the domestic hen (n=5 ± SEM). From Nakamura *et al.* (1979).**

Follicle*	Mean follicle weight (mg)	Hours before ovulation		
		10	4	1
F1	441	0.4 ± 0.1	0.8 ± 0.1	0.4 ± 0.1
F2	359	2.0 ± 0.4	2.1 ± 0.5	1.1 ± 0.1
F3	350	2.7 ± 0.3	3.6 ± 0.5	3.2 ± 0.5
F4	244	2.8 ± 0.4	4.9 ± 0.6	3.5 ± 0.6
F5	158	1.7 ± 0.1	3.1 ± 0.5	3.2 ± 0.5
F6	31	0.7 ± 0.1	1.2 ± 0.2	0.7 ± 0.1

* : Positions of follicle in the hierarchy.

As was mentioned earlier, oestrogens play a major role in the synthesis, transport and deposition of the main egg yolk precursors, vitellogenin and VLDL (section 2.1.3.1), as well as in the onset of egg production (section 2.1.4.1.). They are also involved in the development of secondary sexual characteristics like female-specific plumage, comb, spurs, deposition of pigment in the beak, the shanks, and around the vent. And finally they participate in a wide range of biochemical processes, such as the feedback control on the hypothalamus, stimulation of those areas of the hypothalamus which are associated with sexual behaviour (breeding, oviposition behaviour), development of the medullary bone, softening and spreading of the pubic bones, and increase of blood concentration of calcium, proteins, fats, vitamins, and other substances necessary for egg formation.

The androgens, which are also produced by the thecal cells in the second and third largest follicles, have fewer but indispensable functions in laying hens. They are involved in the development of the comb and all the other secondary sexual characteristics, and have a synergistic action with oestrogens for oviduct growth and medullary bone formation. Androgens are also produced during molting.

In contrast to oestrogens and androgens, progesterone is synthesised by granulosa cells and by more mature follicles. Progesterone is found largely in pre-ovulatory follicles, and to a lower extent in the post-ovulary follicle (Table 6). Progesterone has also a wide range of functions which can be classified into two groups. Firstly, progesterone controls the cellular activities involved in the growth of the oviduct (by acting as an agonist of oestrogens and androgens),

and controls the synthesis of some albumen proteins (by acting in synergy with oestrogens) (section 2.1.3.2.). Secondly, progesterone controls the ovulation and oviposition rhythms by affecting the release of GnRH by the hypothalamus, uterine contractions before oviposition and behaviour at the time of oviposition.

Table 6. Progesterone content of ovarian follicles (ng per follicle) in the domestic hen (n=5 ± SEM). From Nakamura *et al.* (1979).

Follicle*	Mean follicle weight (mg)	Hours before ovulation		
		10	4	1
F1	441	6.6 ± 0.9	9.1 ± 1.0	39.1 ± 2.6
F2	359	4.1 ± 0.5	6.4 ± 1.3	12.8 ± 4.0
F3	350	3.2 ± 0.1	6.2 ± 0.1	8.6 ± 1.0
F4	244	2.8 ± 0.8	5.7 ± 0.5	6.9 ± 0.9
F5	158	2.7 ± 0.3	4.5 ± 0.5	4.3 ± 0.4
F6	31	2.4 ± 0.3	3.7 ± 0.4	3.7 ± 0.4

* : Positions of follicle in the hierarchy.

2.1.4.5. Oestrogens and vitellogenesis

Oestrogens are involved in a wide range of functions and can act on varied tissues. For example, they can stimulate cell proliferation and differentiation in the oviduct, but they can also act on hepatocytes by stimulating the synthesis of yolk precursors. In this section, only the effect of oestrogens on vitellogenesis will be discussed.

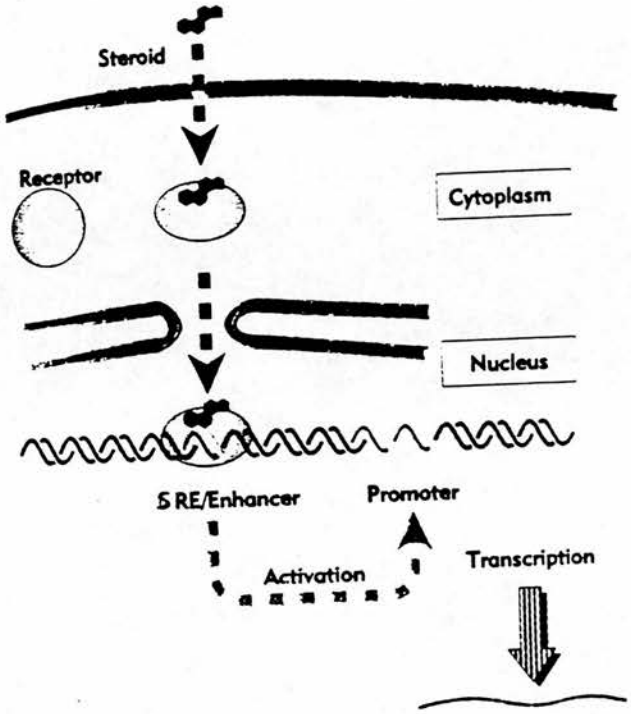
In the blood, oestrogens are found in a free form and, to a larger extent, associated with specific binding proteins such as corticosteroid-binding globulin (CBG), or transcortin, and testosterone-binding globulin (TeBG), or sex-hormone-binding globulin (SHBG). The role of these blood proteins may be simply to transport steroids, which are virtually insoluble in an aqueous medium; or to act as a reservoir from which steroid can be withdrawn at times of need; or to increase the half-life of the steroids. Only the free hormone is biologically active, and as reviewed by Giorgi (1980), most evidence indicates that the hormone enters the cell by passive diffusion, is equally able to enter all cells, but is accumulated in target cells by virtue of its association with specific receptor proteins (see section 2.1.5.2.).

Once oestrogens have entered the hepatocyte, they bind to an intracellular oestrogen receptor. Such receptors were first characterised by Toft and Gorski (1966) in the rat uterus. It is widely believed that, in the target cell cytoplasm, the receptor exists in the 4S form and that unbound receptor is a cytoplasmic protein which, after binding the hormone, acquires an affinity for the nucleus into which it then moves. However, studies by King and Greene (1984) and Welshons *et al.* (1984) have cast considerable doubt as to whether the oestrogen receptor is ever found in the cytoplasm at significant levels. Other authors (Jensen *et al.*, 1982) appeared to show a perinuclear localisation for the receptor. Knowler and Beaumont (1985) have therefore suggested dropping the term *cytoplasmic* receptor in favour of the terms *unfilled* or *unoccupied* receptor.

The formation of the oestrogen-oestrogen receptor (O-OR) complex activates the receptor which then exhibits a greater affinity for nuclear components. This is believed to be caused by an allosteric effect (Yamamoto and Alberts, 1974) and is accompanied by an increase in the sedimentation coefficient from 4 to 5 S. These two processes are separable and have respectively been called activation and transformation (Gschwendt and Kittstein, 1980). Activation is a temperature-dependent process which involves the dissociation of subunits and/or conformational changes (Müller *et al.*, 1983), phosphorylation (Shyamala and Leonard, 1980), proteolysis of the receptor (Puca *et al.*, 1977) and removal or addition of cytosolic factors (Sato *et al.*, 1979). Transformation is a dimerisation process which occur in the cytoplasm or nuclei and which does not involve the addition of other entities (Müller *et al.*, 1983) but involves another molecule as well as the hormone-receptor complex (Thampan and Clark, 1981). Receptor replenishment is assured by both *de novo* synthesis and recycling (Sarff and Gorski, 1971) and receptor turnover reflects the activity of the type of oestrogens (short acting/long-acting).

Once the O-OR complex has moved into the nucleus (Figure 5), the receptor recognises a specific consensus sequence that identifies the oestrogen response element (ORE). The ORE is typically located in an enhancer in the general vicinity of a gene that responds to oestrogens. When the O-OR complex binds to the enhancer, the nearby promoter is activated, and transcription initiates there. Enhancer activation provides the general mechanism by which oestrogens regulate target genes.

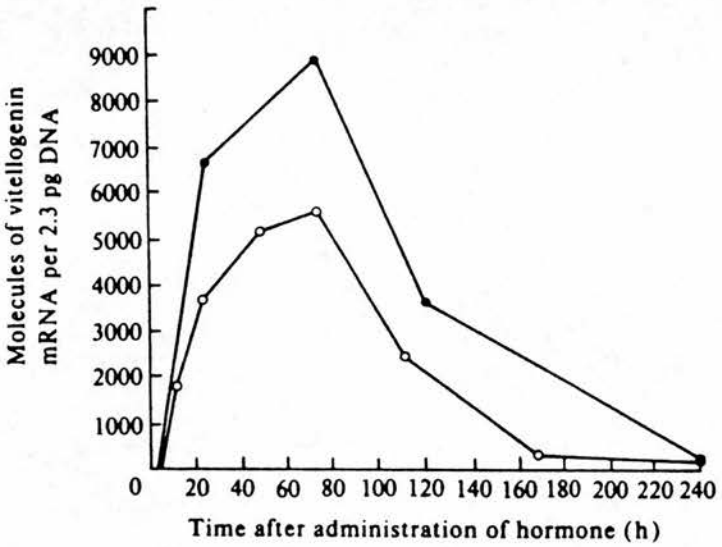
Figure 5. Oestrogens regulate gene transcription by causing their receptor to bind to an enhancer whose action is needed for promoter function (From Lewin, 1995).



When chicks are injected with oestrogens, accumulation of vitellogenin mRNA is observed in the liver after a lag of 6 to 10 h. Secondary stimulation causes synthesis without a lag (Jost *et al.*, 1978). There are at least two different species of vitellogenin mRNA but only the major species is transcribed in response to oestrogen. The other is synthesised constitutively (Cozens *et al.*, 1980). Determination of the amounts of mRNA in the liver of treated male roosters showed that the peak of the response is reached three days following injection, when there are 6,000 to 7,000 molecules per liver cell (Jost *et al.*, 1978). In general, there is a good correlation between the rate of vitellogenin synthesis and the level of vitellogenin mRNA. This is good evidence that for vitellogenesis, as for other steroid-hormone-responsive systems (O'Malley and Means, 1974), regulation occurs at the level of mRNA supply rather than at the level of its translation. The level of vitellogenin mRNA and the rate of vitellogenin synthesis decline exponentially. The mRNA has a longer than average half-life which has been estimated to be 29 h in rooster liver (Burns *et al.*, 1978). If the birds are given a second injection of oestradiol the rate of vitellogenin synthesis rises more rapidly than during the primary response and this seems to be the result of a more rapid accumulation of mRNA during the second response since there are nearly twice as many mRNA molecules present per cell at the height of the secondary response as there are at the height of the primary response (Figure 6) (Deeley *et*

al., 1977). The implication of these results is that oestrogen produces long-lived changes in liver cells which result in a more rapid activation of the vitellogenin gene. This could be due to a permanent increase in the number of oestrogen receptors per cell (Westley and Knowland, 1978) or to a stable alteration in the structure of the gene.

Figure 6. Rates of accumulation and decay of vitellogenin mRNA after primary and secondary hormonal stimulation. Empty circles, molecules of vitellogenin after primary stimulation. Full circles, molecules of vitellogenin after secondary stimulation. From Deeley *et al.* (1977).



The action of oestradiol on the liver causes an increase in the overall rate of protein synthesis (Clemens, 1974) and there is a specific increase in the synthesis of secreted protein. The bulk of this secreted protein is of course vitellogenin, which represents 10 to 15% of protein synthesis in the liver (Wetekam *et al.*, 1975). Simultaneously there is a distinctive change in the size distribution of polysomes in the liver. There is a definite increase in the large polysome fraction of oestrogen-stimulated liver and indirect immuno-precipitation of polysomes has shown that almost all of them were membrane bound (Bos *et al.*, 1972) as would be expected for the synthesis of a secretory protein. Oestrogen stimulation was also shown to increase the synthesis of ribosomal RNA, the capacity of the ribosome to synthesise proteins and the activity or concentration of the peptide chain elongation factors during the period when protein synthesis is increased (Clemens and Tata, 1973).

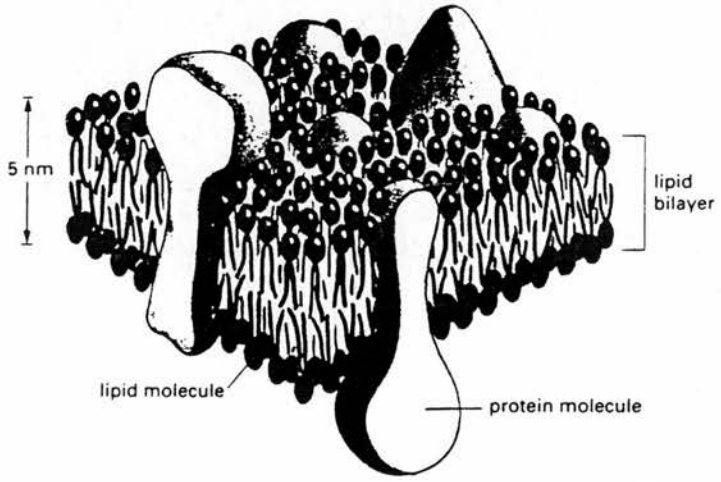
2.1.5. Importance of membranes in egg formation

2.1.5.1. Structure and properties of membranes

The plasma membrane is a selectively permeable barrier between the cell and the extracellular environment. Inside the cell, the membranes of the endoplasmic reticulum, Golgi apparatus, mitochondria, and other membrane-bound organelles in eucaryotic cells maintain the characteristic differences between the contents of each organelle and the cytosol. Despite their differing functions, all biological membranes have a common general structure : each is a very thin film of lipid and protein molecules, held together mainly by noncovalent interactions. Cell membranes are dynamic, fluid structures, and most of their molecules are able to move about in the plane of the membrane. The lipid molecules are arranged as a continuous double layer of 5 nm thick (Figure 7).

The lipid bilayer provides the basic structure of the membrane and serves as a relatively impermeable barrier to the passage of most water-soluble molecules. Protein molecules "dissolved" in the lipid bilayer mediate most of the other functions of the membrane, transporting specific molecules across it, for example, catalysing membrane-associated reactions, such a ATP synthesis. In the plasma membrane some proteins serve as structural links that connect the membrane to the cytoskeleton and/or to either the extracellular matrix or an adjacent cell, while others serve as receptors to detect and transduce chemical signals in the cell's environment. As would be expected, cell membranes are asymmetrical structures: the lipid and protein compositions of the outside and inside faces differ from one another in ways that reflect the different functions performed at the two surfaces of the membrane.

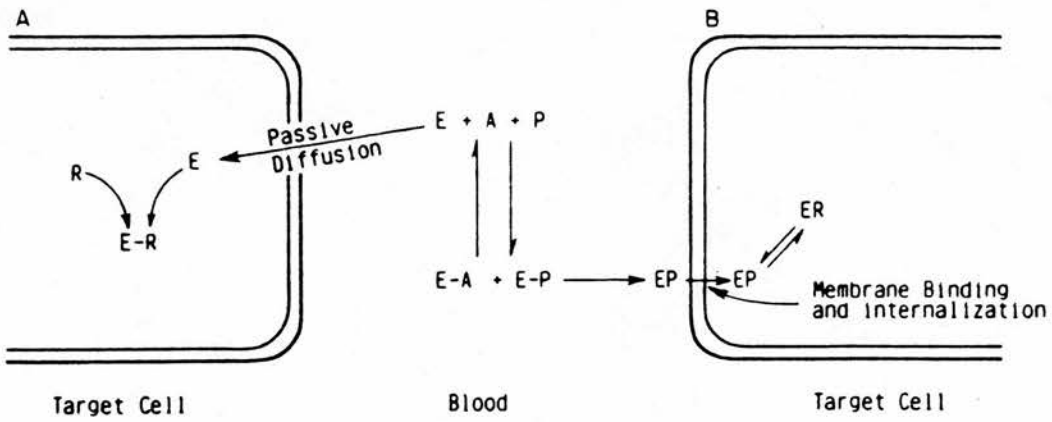
Figure 7. Schematic drawing showing three dimensional views of a cell membrane. From Alberts *et al.* (1994).



2.1.5.2. Membranes and oestrogen transport

As was mentioned in section 2.1.4.5., most evidence suggests that oestrogen enters the cell by passive diffusion and is accumulated in target cells by virtue of its association with specific receptor proteins. This view of the transport of steroids and their entry into target cells is not, however, universal. Milgrom *et al.* (1973) have presented evidence for facilitated transport of oestrogen, and Baulieu (1975) and Uriel *et al.* (1976) have postulated a protein-mediated step in the entry of oestrogen into uterine cells. More recently, Szego and Pietras (1981) reviewed their considerable evidence that there are oestrogen-binding sites on the uterine cell membrane and have suggested that these may be involved in cellular uptake. Siiteri *et al.* (1982) proposed a model in which hormone complexed with specific plasma globulins is bound at specific sites on the cell membrane (Figure 8). The bound complexes are then internalised and supply hormone to the receptor or even turn into the cytoplasmic receptor. This model has however been questioned as some species such as rodents do not have the sex hormone-binding globulin.

Figure 8. Models for the entry of oestrogen into its target cells. A, commonly accepted model; B, alternative model of Siiteri *et al.* (1982). E, Free hormone; E-A, hormone bound to albumin; E-P, hormone bound to plasma globulin; E-R, hormone bound to cytoplasmic receptor protein.

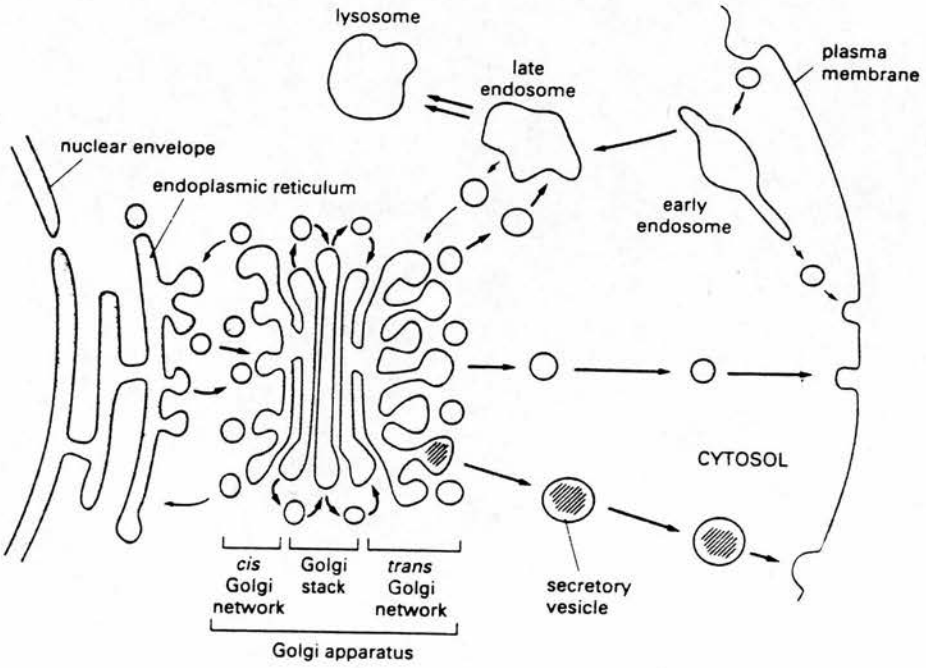


2.1.5.3. Membranes and vitellogenin secretion

Newly synthesised vitellogenin enters the biosynthetic-secretory pathway in the endoplasmic reticulum (ER) by crossing the ER membrane from the cytosol (Figure 9). Subsequent transport, from the ER to the Golgi apparatus and from the Golgi apparatus to the cell surface and elsewhere, is mediated by transport vesicles, which transfer vitellogenin from membrane to

membrane or from lumen to lumen (or to extracellular space) by cycles of vesicle budding and fusion. The transfer of vitellogenin from an organelle to the other and finally its export from the cell is made possible by the structure and fluidity of the intracellular and cellular membranes. Secretion of vitellogenin by cockerel hepatocytes after oestrogen stimulation has been studied by Kami and Stoward (1985) who carried out protein A-gold immunocytochemical studies. Labelling was detected over the cisternae of the rough endoplasmic reticulum (RER), the Golgi apparatus, the immature secretory vacuoles (ISV) including condensing vacuoles, and the mature secretory vacuoles (MSV). Counts of the gold labels demonstrated that the highest density of labelling was obtained on the 4th day and that the increasing concentration of gold labels progressed in order RER < Golgi < ISV < MSV and identified the secretory pathway of the protein. Autophagic activity was also found in lysosomes and its labelling intensity increased daily. Thus, the shuttle of intracellular secretory proteins between secretory vacuoles and lysosomes may occur to allow their degradation (crinophagy).

Figure 9. The intracellular compartments of the eucaryotic cell involved in the biosynthetic-secretory and endocytic pathways. From Alberts *et al.* (1994).



2.1.5.4. Membranes and egg yolk precursors uptake by oocytes

The delivery of both vitellogenin and VLDL particles from the surface of the vitelline

membrane into the yolk is ensured by the presence of specific receptors on the surface of the ovum that recognise apolipoprotein-B, which is a protein included in the VLDL particles, and vitellogenin (Etches, 1996). The receptor is a 95 kDa molecule embedded in the vitelline membrane which binds to specific regions of the vitellogenin and apoprotein molecules. When segments of membrane are loaded with VLDL particles, the section forms a membrane-bound vesicle that is endocytosed inside the yolk. Within minutes after the vesicle is transported into the oocyte, VLDL particles are removed from the membrane. Apo-B is cleaved into four smaller proteins and vitellogenin is cleaved into phosvitin and lipovitellin, by an enzyme produced by the developing oocyte, cathepsin D. As the apoproteins are cleaved, the particles coalesce into larger spheres of up to 150 μm and the yolk granules are formed around phosvitin and lipovitellin. The membrane surrounding the vesicle is used to contain the yolk sphere and excess membrane is presumed to be recycled to the vitelline membrane. The integration and rearrangement of the plasma VLDL particles into fully formed yolk spheres is completed within 20 minutes. This remarkable mode of uptake of vast quantities of material is aided by extensive projections of the vitelline membrane into the developing yolk to increase the surface area available for endocytosis.

2.2. Influence of heat stress in laying hens

The tremendous increase in egg productivity which occurred over the last half century (section 2.1.) has come about as a result of selective breeding which has generally been carried out in temperate countries and under ideal husbandry conditions. This selection has, however, led to a decrease in the ability of birds to adapt to changes in environmental conditions and a greater susceptibility to stresses, particularly heat stress. As the major expansion in the egg production industry occurs in the hot regions of the world (section 2.1.), heat stress constitutes an important issue in the egg production industry.

Some differences exist between thermoregulation in birds and mammals, both in mechanisms and control systems. These variations are mainly due to the organisation of the general biological functions (e.g. the respiratory system). Even if the control of body temperature is very efficient and allows the survival of some species in extreme conditions, for most commercial birds, an increase of ambient temperature above 25°C leads to a surpassing of thermoregulation capacities and consequent heat stress. This can have a severe adverse effect on egg production (El Boushy and Van Marle, 1978).

2.2.1. Stress and heat stress

Stress is a general subjective term used to describe the sum of non-specific responses or defence mechanisms of the body when faced with abnormal or extreme demands (Selye, 1950). Many factors including environmental, nutritional and pathological disturbances can generate a state of stress. Therefore stressful conditions evoke a combination of behavioural, biochemical and physiological adaptations and generally result in a reduction of production performance.

Heat stress is a physiological stress resulting from an imbalance between the heat produced by the body (thermogenesis) and the heat loss from the body (thermolysis). For mild and brief increases of ambient temperature, regulatory processes can re-establish the equilibrium or homeostatic state. But when the changes are too severe, these responses are insufficient and core temperature inevitably increases. Under these circumstances, a wide variety of behavioural, physiological, molecular and neuroendocrine responses are initiated to try to maintain body temperature within the normal limits.

2.2.2. Heat stress and thermoregulation

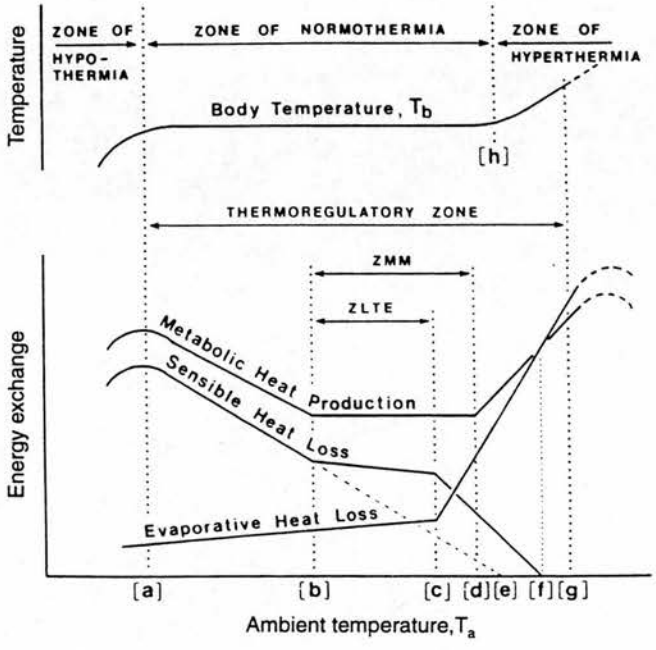
2.2.2.1. Heat stress and body temperature

In contrast to the poikilothermic reptiles from which they were derived, birds, like mammals, are homeothermic. In well-fed laying hens that are neither dissipating heat to the environment nor gaining heat from it, body temperature can be maintained fairly constant, 41.9 ± 0.5°C (King and Farner, 1961). The range of ambient temperature in which no heat is dissipated or gained, defined as the zone of normothermia, varies between 10 and 20°C according to El Boushy and Raterink (1985) (Figure 10).

As this body temperature is higher than in mammals, birds are less sensitive to heat stress (Meltzer, 1983). However if the ambient temperature rises beyond the upper critical temperature, heat production must be reduced or heat must be lost to prevent body temperature increasing (Hafez, 1968). Two centres are responsible for heat balance control : the heat production centre is located in the lateral hypothalamus, whilst the heat loss centre is located in the anterior hypothalamus (Amakiri and Heath, 1985). Different factors can influence the susceptibility of laying hens to heat stress, such as age, feathering or fatness state. Heavier birds generally have more problem with heat stress since they have less surface for heat

dissipation per unit weight (Teeter, 1994).

Figure 10. Generalised schematic diagram illustrating T_b (body temperature) and partitioning of energy exchange through a wide range of T_a (ambient temperature). From Hillman *et al.* (1985).



The zones and points are : ZMN, zone of minimum metabolism; ZLTE, zone of least thermoregulatory effort; point [a] lower critical temperature; point [b], critical temperature; point [c], temperature at which intense evaporative heat loss begins; point [d], upper critical temperature; point [e], T_a which equals normothermic T_b; point [f] where sensible heat loss is zero because metabolic heat production equals evaporative heat loss; point [g], critical thermal maximum; point [h], point of incipient hyperthermia.

2.2.2.2. Decrease in thermogenesis

As ambient temperature rises, all the metabolic reactions occurring within a cell are passively increased (North, 1972) (Figure 10). Thermogenesis is therefore boosted during heat stress. To compensate, general metabolism is reduced by about 25 to 35% and rapidly reaches a low level of maintenance metabolism or basal metabolism (Ruckebrush *et al.*, 1991). Lipid and carbohydrate metabolisms are reduced through a decrease in the activity of the thyroid gland (section 2.2.4.3), physical activity is reduced (section 2.2.3) and voluntary food is diminished (Otten *et al.*, 1989). The reduction in voluntary food intake results in a lower energy intake and reduced heat production by the digestive tract. As this is generally accompanied by a decreased celiac blood flow (Bottje and Harrison, 1987), food digestibility is reduced. The

reduction of food intake has been studied by a few authors and estimated at 5% per additional °C between 32 and 38°C compared to only 1 to 1.5% between 20 and 30°C (Polin, 1983; Deaton *et al.*, 1986).

2.2.2.3. Increase in thermolysis

As laying hens do not have sudoriparous glands, heat can be dissipated either by non-evaporative channels (convection, conduction and radiation) or by evaporative channels (water evaporation through skin or respiratory tract or by both). These methods of dissipating heat are highly influenced by the physical characteristics of the animal, such as its body surface area, type and thickness of skin and body covering, thermal conductivity of the tissues peripheral to the blood flow (Hsia, 1990).

The elimination of heat through non-evaporative channels is positively correlated to the gradient of temperature between the body surface and the environment and occurs in highly vascularised and featherless zones such as the comb, barbs, legs (Richards, 1971). Although very efficient in temperate conditions when 75% of the heat produced is eliminated this way (El Boushy and Van Marle, 1978), this mechanism only represents less than 20% of total heat loss when the ambient temperature exceeds 35°C (Sturkie, 1986). The other way of losing heat, which relies on the evaporation of water from the skin and respiratory surfaces, plays the most important role in heat loss under high environmental temperature (60% of total heat loss at 32°C, Ota *et al.*, 1953).

To increase the amount of water evaporated through the lungs, the respiratory rate is accelerated (Bligh *et al.*, 1976). When the body temperature reaches 42°C, panting is initiated (Hillman *et al.*, 1985). This response is characterised by a tremendous increase of respiratory rate and decrease of tidal volume (Dawson, 1982) and is controlled by receptors in spinal chord and hypothalamus and by afferent vagal system (Woods and Whittow, 1974; Dawson, 1982). As the avian lung is a very effective gas exchange organ, twice as much carbon dioxide can by removed from the pulmonary blood in a bird than in a mammal of the same size (Ramirez and Bernstein, 1976). However, when the environmental temperature gets too high, the effectiveness of the panting process is limited as the muscular effort generates heat and as the increased exchange of gases leads respiratory alkalosis (section 2.2.4.1.).

2.2.3. Heat stress and behaviour

During thermal stress, birds alter their behaviour to help maintain body temperature within the normal limits. Behavioural adjustments can occur rapidly and at less cost to the bird than most physiological adjustments (Lustick, 1983), although they are preceded by the molecular response to heat stress that is mediated by the heat-shock proteins (section 2.2.4.4.).

As ambient temperature increases, birds reduce their physical activity and muscular heat production (Mc Farlane *et al.*, 1989). They look for comfortable positions (sitting instead of standing up, Van Kampen, 1976) and appropriate places (shaded, ventilated and humid, Sturkie, 1986). They also reduce their food consumption to limit energy intake and heat production by the digestive tract (Li *et al.*, 1992) and increase water consumption to compensate from water loss through evaporative cooling (Mench, 1985). Spreading of wings and featherless zones (comb, barbs, legs) and loss of feathers also occur spontaneously to favour the contact of these zones with a cold conductive medium, such as the ground, fresh air or water.

2.2.4. Heat stress and physiology

2.2.4.1. Respiratory changes

During heat stress, and to increase evaporative heat loss, the respiratory rate is accelerated and birds start to pant (section 2.2.2.3.). This process, which allows dissipation of heat at the surface of the mouth and respiratory passageways, may also affect pulmonary gas exchange, blood gas transport, tissue gas exchange, cellular metabolism and acid-base balance (Kazemi and Jhonson, 1986). As the amount of air expired is increased, the partial pressure (pp) of gases are modified, $pp0_2$ is increased, $ppCO_2$ is reduced and hypocapnia occurs. This generally leads to a rise in the pH (and to a decrease in the H^+ concentration) in the blood which generates an acid-base imbalance or respiratory alkalosis (Marder and Arad, 1989).

Recent studies have shown that two different forms of panting may be observed in heat stressed laying hens. Simple panting has been described as the most common pattern of thermal panting practised by birds. It generally results in a 20 to 30 time-increase of breathing frequency and in a reduction of tidal volume to the tracheal space. This form of panting can go on for several hours and can result in a slight respiratory alkalosis which is fully overcome after 40 minutes

panting (Bech and Johansen, 1980). Flush out panting, which shows similar characteristics as the simple panting, is interrupted at regular intervals by shortlasting sequences of deeper breaths, which serves evaporative cooling (Marder and Arad, 1989).

In addition to producing alkalosis and hypocapnia, thermal panting has additional disadvantages for the birds. Not only can it increase heat production by respiratory muscles during hyperventilation, but it also prevent birds from drinking and feeding while cooling their body and might affect egg shell formation through changes in blood pH and reduction in plasma calcium levels (Odom *et al.*, 1986).

2.2.4.2. Circulatory changes

During panting, blood flow to the upper respiratory tract more than doubles to provide water for evaporation, whilst blood to some other parts of the body, such as digestive organs, is reduced (Akester, 1984; Bottje and Harrisson, 1987). Loss of heat also occurs by increasing the blood flow to, and thus the heat loss from, those parts of the body which are not feathered and it is generally agreed that peripheral circulation is a modifier of heat transfer to the environment. Dilation of capillary vessels during heat stress allows greater flow of warm blood to exposed parts of the body and thus more heat can dissipated by convection and radiation mechanisms. This is particularly true in the case of laying hens, as their unfeathered feet are sites of high thermal conductance to the environment and are a potential asset for heat dissipation (Hillman *et al.*, 1982). As a result, in heat stressed birds, blood pressure declines, precipitously at body temperature above 45°C. In the terminal stages of hyperthermia, cardiac output also decreases. Thus circulatory failure is a contributing cause of death in the bird at extremely high temperatures (Hafez, 1968).

2.2.4.3. Hormonal changes

As a bird attempts to cope with heat stress, an intricate series of changes mediated by many hormonal systems is initiated. The relative importance of each of these systems and the extent to which they are called upon depend on the severity of the heat stress. Hormones which regulate thermogenic processes include the neurohypophyseal hormones, arginine vasotocin (AVT) and mesotocin (MT), growth hormone (GH), and thyroid hormones. During heat stress, the increased concentrations of AVT and GH are believed to play a role in heat dissipation

(Wang *et al.*, 1989; John *et al.*, 1975). Indeed, both of these hormones have been shown to stimulate the mobilisation of free fatty acids and thus sustain the high respiratory muscles activity initiated by panting (George and Berger, 1966). The increase of AVT, which also suppresses MT release, favours the conservation of body fluids in the organism during heat stress (Wang *et al.*, 1989). The concentrations of the two active forms of thyroid hormones (T_4 and T_3) are depressed during heat stress, thus reducing the metabolism of lipids and carbohydrates (May *et al.*, 1986) and improving heat tolerance (Hahn *et al.*, 1966).

Another group of hormones affected by heat stress are the adrenal hormones. Heat stress stimulates the release of ACTH by the pituitary gland which, in turn, stimulates the release of corticosterone, aldosterone and catecholamines. ACTH, corticosterone, aldosterone and catecholamines have widespread effects on many target tissues throughout the body that delay hyperthermia. Dilatation of vessels results from the formation of arterio-veinous anastomoses and vascular resistance is reduced by adrenagic control (Wolfenson, 1983). However the surge of corticosterone in response to heat stress can only be maintained for a short period of time and the concentration of corticosterone drops below pre-exposure level within a few hours (Edens, 1978). This drop is accompanied by low plasma concentrations of glucose, phosphate and Na^+ and elevated plasma pH resulting in an acute adrenal cortical insufficiency which, in conjunction with a massive secretion of catecholamines, brings about cardiovascular failure and death. Changes in the plasma concentrations of corticosteroids and ACTH affect the lymphoid tissues and consequently the ability of birds to mount an immune response. A diminution in the mass of the thymus, spleen and bursa of Fabricius, number of circulating lymphocytes and an increase in neutrophilic or heterophilic granulocytes have been reported following administration of corticosteroids.

Melatonin (N-acetyl-5-methoxytryptamine), which is a major pineal hormone, also affects thermoregulation in birds (John and George, 1991). In the presence of high concentrations of melatonin, heat dissipation by peripheral tissues is enhanced by vasodilatation and blood flow, particularly to the foot. Furthermore, melatonin may act centrally by lowering the set-point of the main "thermostat", which is believed to be present in the hypothalamus (John and George, 1991).

Finally, heat stress has and effect on reproductive hormones. During heat stress, LHRH level is reduced (Donoghue *et al.*, 1989) and so are the preovulatory surges of plasma LH and progesterone (Novero *et al.*, 1991). Indeed, corticosterone is known to have a suppressive

effect on circulating LH levels in birds (Pettite and Etches, 1988) and the high concentration of circulating corticosterone during thermal stress (Donoghue *et al.*, 1989) could promote the decline of LHRH.

2.2.4.4. Heat-shock proteins

As in all known organisms, elevated body temperature in birds increases the synthesis of heat-shock proteins (reviewed by Nover, 1991) which associate with a variety of proteins to affect their conformation and location or protect them from degradation. The most commonly found forms of heat-shock proteins have relative molecular masses of approximately 70 and 90 kDa and are referred to as HSP70, HSP90. Members of the HSP70 family bind to immunoglobulin heavy chains, clathrin baskets, deoxyribonucleic acid (DNA) replication complexes, adenosine triphosphate (ATP) and may prevent precipitation of the cellular proteins that have been denatured during heat stress (Finley *et al.*, 1984). HSP90, whose family members are abundant at normal temperatures, interacts with steroid hormone receptors and apparently masks the DNA-binding region of the receptor until the receptor has bound to the appropriate steroid hormone. Transcription of heat-shock protein-encoding genes is regulated by a heat-shock factor (HSF), which interacts with a conserved DNA sequence, the heat-shock element (HSE). The binding of significant amounts of HSF to HSE only occurs after a heat shock and HSF can also bind to other regions of the genome, and may play a role in suppressing transcription of other genes during the period of stress (Westwood *et al.*, 1991).

2.2.5. Heat stress and membrane damage

Exposure to high ambient temperatures increases free radical and reactive oxygen species (ROS) burden in the body (Bendich, 1993). Free radicals are defined as any molecules capable of independent existence that contains one or more unpaired electrons, i.e. electrons present alone in atomic or molecular orbitals. The presence of unpaired electrons is usually associated with decreased stability and increased reactivity. Free radicals can derive either from normal, essential metabolic processes, such as phagocytosis, exercise, mitochondrial function, or from external sources such as cigarette smoke, radiation or stress (Fürst, 1996). Because antioxidant defences are not 100% efficient, free radical reactivity can damage all types of cellular macromolecules, including proteins, carbohydrates, lipids and nucleic acids. Possibly the most reactive radical species to form in living organisms is the hydroxyl radical, $OH^{\cdot}$. Important biological sources of $OH^{\cdot}$ arise from the decomposition of hydrogen peroxide (H_2O_2)

by the Fenton reaction (Eq. 1), by the interaction of superoxide and hydrogen peroxide through the Haber-Weiss reaction (Eq. 2) and by the interaction of transition metals with hydrogen peroxide (Eq. 3).

(Eq. 1) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$ (Fenton Reaction)

(Eq. 2) $O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + OH^{-} + OH^{\cdot}$ (Haber-Weiss)

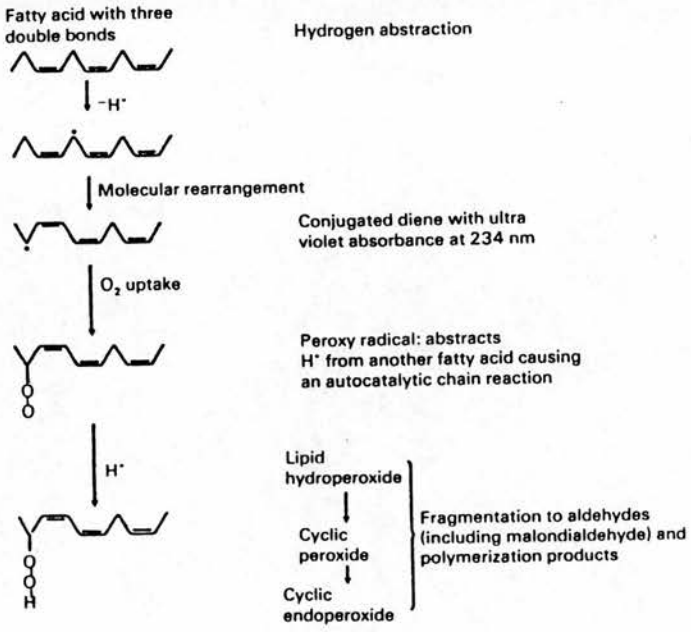
(Eq. 3) $Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + OH^{\cdot} + OH^{-}$

Although another oxygen radical, $O_2^{\cdot-}$, was demonstrated to damage biomolecules, radiation studies pointed out that it is the least reactive of any of the ROS. If pure lipids are exposed to pure $O_2^{\cdot-}$ radical, no reaction is seen. The same is true of H_2O_2 at the sort of concentration likely to be present in living organisms. The idea therefore grew that the biological cell damage done by $O_2^{\cdot-}$-generating systems is not due directly to $O_2^{\cdot-}$ but occurs because the $O_2^{\cdot-}$ participates in formation of $OH^{\cdot}$ (Halliwell, 1987). This radical will attack almost any biological molecule that it is generated next to. If $OH^{\cdot}$ is formed close to a membrane, it tends to attack the polyunsaturated fatty acid side-chains in the membrane lipids (see section 2.1.5.) and remove an atom of hydrogen ($H^{\cdot}$) to form water. This reaction leaves behind in the membrane another type of radical species, a carbon-centred radical ($L^{\cdot}$). The latter can undergo reaction with molecular oxygen to form a third type of radical species, the peroxy radical ($LOO^{\cdot}$), with the concomitant release of malondialdehyde (MDA). Peroxy radicals can react with another membrane fatty acid side-chain and become converted themselves into lipid peroxides. This is achieved by further $H^{\cdot}$ abstraction, forming another carbon-centred radical that can react with more O_2 and carry on the reaction. Hence $OH^{\cdot}$ attack on a membrane system can start off a radical chain reaction known as lipid peroxidation (Halliwell and Gutteridge, 1989) (Figure 11).

A lipid peroxide is obviously more polar than anything that should be present in the hydrophobic interior of a biological membrane. Lipid peroxidation diminishes membrane fluidity, increases non-specific permeability to ions (e.g. Ca^{2+}) and may inactivate membrane-bound enzymes. Further, in the presence of various kinds of metal complexes, lipid peroxides decompose to produce many different fragments that include more radicals, hydrocarbon gases and several aldehydes that are highly cytotoxic even in minute amounts. The release of MDA, which can generate crosslinking of proteins, can inactivate membrane bound enzymes and physical changes within the membrane can alter tertiary structure of membrane proteins. There

is evidence that Na^+/K^+ ATPase activity is disrupted by lipid peroxidation (Thomas and Reed, 1990); such disruption could contribute to an inability of membranes to maintain the electrical gradient required for cell viability. Thus an attack of $OH^\cdot$ on a membrane lipid starts off a series of radical chain reactions that can severely damage the membrane. The reactions of $OH^\cdot$ with other molecules, such as thiamine in DNA, can proceed similarly; there is an initial radical formed, which then reacts with O_2 and goes on to bring about further reactions.

Figure 11. The chain reaction of lipid peroxidation. From Halliwell (1987).



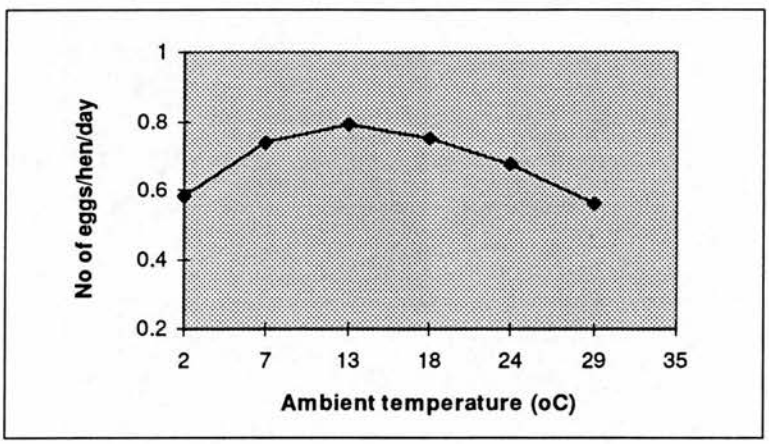
2.2.6. Heat stress and egg production

During heat stress, a wide range of mechanisms are involved to limit the increase in body temperature, e.g. reductions in basal metabolism, feed intake, intestinal absorption of nutrients, adjustments in behaviour and physiology (section 2.2.2., 2.2.3., 2.2.4.), but egg production is affected as soon as the ambient temperature exceeds 20 or 25°C. The reduction of production performance in heat-stressed laying hens has been well documented (de Andrade *et al.*, 1977; Miller and Sunde, 1975; Deaton *et al.*, 1981). It has also been observed that egg production is more affected under constant high temperatures than in cyclic high temperatures (Miller and Sunde, 1975).

2.2.6.1. Number of eggs produced

When the ambient temperature increases, the number of eggs produced decreases (Figure 12). According to Ota (1960), the number of eggs produced at 29°C represents 72% of production at 13°C. The magnitude of the decrease was also shown to be more pronounced at higher ambient temperatures. Smith and Oliver (1972) demonstrated a decrease of 3.6% in egg production between 29.5 and 32°C and a decrease of 18% between 32 and 35°C. The magnitude of the reduction in egg production was also shown to increase with the age of the birds. Uzu (1985) found a reduction of egg production of 9% in birds aged 24 weeks and of 13% in birds aged 52 weeks when the ambient temperature was increased from 20 to 30°C.

Figure 12. Egg production at various house temperatures. From Card and Nesheim (1972).



2.2.6.2. Egg weight

Mean egg weight was also decreases when the ambient temperature increases (Figure 13). From the curve established by Smith and Oliver (1972), the relationship between ambient temperature and mean egg weight was determined :

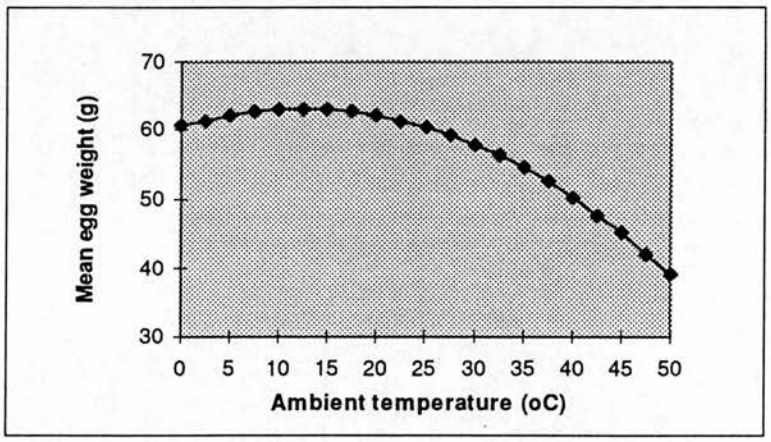
$$\text{Egg weight (g)} = 59.60 - 1.34\,(0.2\,T - 16) - 0.313\,(0.2\,T - 16)^2$$

where T = ambient temperature in °F

Hermes *et al.* (1983) demonstrated that the effect of heat stress on mean egg weight was affected by the body weight of the birds. For a variation of ambient temperature from 20 to 30°C, the mean egg weight was reduced by 2.3 g for birds aged 24 weeks and by 5.6 g for birds

aged 52 weeks. Although the decrease in egg weight was shown to affect both the albumen and yolk, Ahvar *et al.* (1981) demonstrated a greater decrease in yolk weight (13.5%) than in the albumen weight (7.2%) in birds aged 55 weeks and exposed to a variation of ambient temperature from 20 to 32°C.

Figure 13. Effect of air temperature on egg weight. From Smith and Oliver (1972).



2.2.6.3. Egg shell quality

Thickness and weight of the shell have been shown to be affected by the ambient temperature (Ahvar *et al.*, 1981). On average, for an increase in ambient temperature from 20 to 32°C, the thickness of the shell is reduced by 9.5% and its weight by 14.5%. Concurrent with the decrease in shell weight, reduction of the ratio of shell weight : egg weight has been demonstrated by Ahvar *et al.* (1981). Miller and Sunde (1975) also found a greater deformability of eggshells in hot climate. The reduction of shell thickness at high temperature has been attributed to reduced calcium intake as a result of the reduced feed intake and to the occurrence of respiratory alkalosis (section 2.2.4.1.).

2.2.7. Attempts to reduce the detrimental effects of heat stress

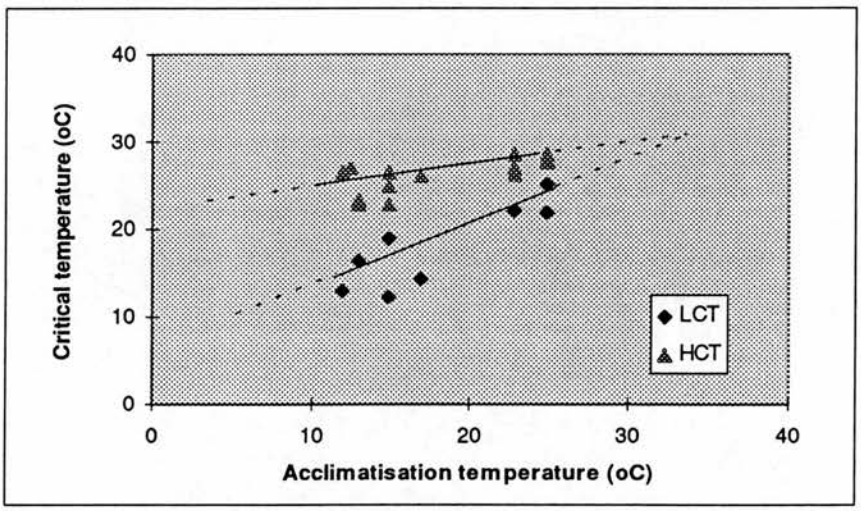
2.2.7.1. Non-nutritional attempts

2.2.7.1.1. Acclimatisation

Acclimatisation to heat stress has been defined as the long-term adaptive physiological adjustments which result in an increased tolerance to continuous or repeated exposure to stress

(Hafez, 1968). Its objective is to increase the upper critical temperature of the zone of thermoneutrality (Figure 14). Using two groups of hens acclimatised either to summer or winter conditions, with an average difference of 10°C between these two seasons, Arieli *et al.* (1980) demonstrated an increase in the upper critical temperature by 3°C. The increase in tolerance to heat is reflected in lower body temperatures, higher panting rates and decreased evaporative water loss which are consequences of the lower temperature of the evaporative surfaces.

Figure 14. Effect of acclimatisation on lower and upper critical temperatures in laying hen. From Arieli *et al.* (1980).



Since only the legs and the comb offer surface for heat exchange, there is a limited scope for increasing heat loss by vasodilatation. Therefore acclimatisation is mainly achieved by reducing heat production (Sykes and Fataftah, 1986). Reduction in metabolic rate, and therefore heat production, is generally observed during acclimatisation and thyroid activity is reduced (Falconer, 1971). Strain differences in the response to heat stress have also been reported (Khan, 1992), although it was not concluded whether they were solely a reflection of body size and metabolic rate, or of some other genetically determined character.

2.2.7.1.2. Ventilation

In hot countries, poultry houses are generally of an open type. The natural ventilation, which depends on the orientation of the shed and on the density of the vegetal environment around the shed, has a strong influence on the ambient temperature inside the shed. To improve the

control of the temperature within the shed, El Boushy and Van Marle (1978) promoted the use of a dynamic ventilation to assure a better air movement, a satisfactory concentration of oxygen, humidity, ammoniaque and dioxide carbon in the air and an optimal temperature. During heat stress, the heat dissipated by the birds can therefore be eliminated more rapidly and the temperature maintained to an appropriate level. North (1972) demonstrated that the optimal air flow at 26.7°C was 0.05 m^3 per minute. Drury and Siegel (1966) showed that air speeds of 152 to 177 m per minute allowed birds to stand ambient temperature of 40.6°C and Uzu (1989) showed that an air speed of 25 m per minute increased total egg mass by 9% in birds maintained at 31°C and 75% R.H. According to Sutton (1952), these measures can be combined with cooling of the air entering the shed. Splashing of water on the roof of the shed has been shown to reduce its temperature from 67 to 38°C. The temperature of the air entering a shed can also be reduced when water is vaporised in the air entering in the shed.

2.2.7.1.3. Non-nutritional food additives

Several non-nutritional food additives have been tested for their ability to reduce the harmful effects of high temperature stress. Antibiotics are widely used and have at times been shown to reduce the effects of heat stress. However, reports on their use as anti-stressors are often contradictory (Freeman *et al.*, 1975). Acetylsalicylic acid (aspirin) has also been used against stress. Balog and Hester (1989) demonstrated a reduction in the production of shell-less eggs in layers fed 0.05% acetylsalicylic acid for a period of 4 weeks. Cocciodiostats (monensin, arprinocid, nicarbazin) have been shown to reduce heat-stress mortality (McDougal and McQuistion, 1980) and reserpine, an alkaloid that is extracted from the *Rauwolfia* plant, was found to prevent the rapid loss of CO_2 which occurs during heat stress-induced alkalosis (section 1.2.4.1.) (Edens and Siegel, 1974).

2.2.7.2. Nutritional attempts

In temperate conditions, the relative stability of the environmental conditions in poultry houses is combined with the *ad libitum* feeding of balanced diets. However, in hot countries, manipulations of the nutrient content of diets or feeding programmes are widely used to combat effects of heat stress (Picard *et al.*, 1993).

2.2.7.2.1. Modifications to feeding programmes

As the action of consuming food generates heat, food withdrawal during the hottest hours of the day has become a common management practice to combat heat stress. In a tropical environment, Ramlah and Jaluladin (1989) observed in increase of 4% in egg production and a decrease of 5% in food intake when the birds received 3-hour meals instead of being fed *ad libitum.* Under more severe temperature conditions (32/41°C), Huthail (1992) also demonstrated a significant increase in egg production when food access was suppressed for 11 h per day. In the case of an artificial cyclic hot climate (26°C during the night and a peak of 39°C at 4 p.m.), no difference was however found by Picard *et al.* (1993) between birds fed continuously throughout the day or fed only for 4 h in the morning and 5 h in the evening.

2.2.7.2.2. Distribution of time-limited calcium meals

As a high dietary concentration of calcium has been shown to depress food intake (Hull and Scott, 1969) and to contribute effectively to shell formation for only a few hours per day, when the shell is formed (Sauveur and Mongin, 1974), a technique consisting of providing birds with a separate and temporary source of calcium has been proposed. This technique was found to increase energy intake (Cabrera *et al.*, 1982) and to improve egg production (Uzu, 1988) during heat stress. Another technique, which consists of distributing a diet rich in calcium and poor in phosphorus in the evening and rich in phosphorus and poor in calcium in the morning (Rodriguez, 1991), has been shown to increase energy intake in the morning. This technique is therefore particularly efficient in hot countries as food intake is also greater in the morning as ambient temperature is cooler.

2.2.7.2.3. Modifications to diet composition

- Protein and amino acid

It has long been recommended that, during hot conditions, the percentage of protein or amino acids in layer diets should be increased commensurate with the reduction in food intake (Heywang, 1947). More recent studies, however, did not lend strong support to this practice, and suggested that such adjustments may in fact be detrimental. Proteins have the highest heat increment, carbohydrates are intermediate, and fats the lowest and Fuller (1977) demonstrated that food intake is influenced as much by the heat increment of the diet as it is by its energy

content, particularly under heat stress.

As the economics of a high-protein diet is questionable, examination of critical amino acids may yield a more practical and profitable approach. Lysine and methionine have been recognised to be the two main limiting amino acids in poultry nutrition (Harms and Waldroup, 1962). Austic (1985) suggested that the essential amino acids should be raised for increases of ambient temperature between 20 and 30°C, but not above 30°C because of the reduction of egg production. The latter was questioned by Uzu (1989) who demonstrated that, under high ambient temperatures, the methionine requirement remains high even if egg production is reduced. The importance of the relative equilibrium of the different amino acids in the diet in hot climates was confirmed by Biely and March (1963) who demonstrated that birds fed a diet containing 14% protein and supplemented with both lysine and methionine produced heavier eggs than birds fed the same diet but supplemented with only lysine or only methionine. Similarly, an excess of one or several amino acids above requirement, which increases thermogenesis, reduces food intake and egg production (Waldroup, 1982).

- Energy

It has been known for a long time that metabolisable energy (ME) requirement decreases with increasing temperature. Recent studies have however demonstrated that this is true only within the zone of thermoneutrality. At very low temperatures, birds tend to overeat and at high temperatures they undereat. Above 30°C, feed intake decreases more rapidly and the energy requirements begin to increase. This increase reflects the body's effort to get rid of the extra heat burden caused by high temperature. De Andrade *et al.* (1976) demonstrated the positive effect of a concentration of the diet in energy and nutrients provided that the ratio of energy : proteins is reduced.

However, increasing the energy concentration of a diet in hot and developing countries is a solution which, for economical reasons, is not easily applicable. Picard (1985) showed that, in hot climates, laying hens can benefit from receiving diets with a very low energy content (2,100 kcal ME/kg) containing up to 50% by-products rich in fibres. For a similar energy intake, food consumption was shown to be increased by 30% when the ME content of the diet was reduced from 2540 to 2110 kcal ME/kg, which allows the bird to eat enough non-energetic nutrients (Uzu, 1986).

- Minerals

Different methods involving mineral supplementation in the diet or in the water have been tried in attempts to reduce the detrimental effect of heat stress on egg production, egg weight or eggshell strength. Improved egg production and shell quality have been shown with dietary supplementation with calcium (section 2.2.7.2). In contrast to calcium, a high concentration of phosphorus in the diet has been shown to be detrimental for egg weight and egg shell quality (Daghir, 1987). During heat stress, an increase in the ratio of calcium to phosphorus to a value of 5.8 (3.5% calcium, 0.6% phosphorus) was recommended by Ademosun and Kalango (1973).

Respiratory alkalosis can also be combated by providing a source of anion via feed or water. Teeter and Smith (1986) demonstrated that supplemental ammonium chloride in drinking water of chronically heat-stressed birds returned blood pH to normal and enhanced weight gain. During acute heat stress, provision of ammonium chloride (Branton *et al.*, 1986) or carbonated water (Bottje and Harrison, 1985) was also shown to decrease blood pH. Provision of carbonated water was also shown to improve egg production, eggshell thickness and tibia bone breaking strength during heat stress (Odom *et al.*, 1985; Koelkebeck *et al.*, 1993).

- Vitamins

Ascorbic acid (vitamin C) has been the most studied hydrosoluble vitamin in relation to ambient temperature. There is some evidence that, under conditions of high environmental temperatures, some birds are not able to synthesise sufficient vitamin C to replace the severe losses of this vitamin that occur during stress. As early as 1961, Thornton showed that blood vitamin C decreased with an increase in environmental temperature from 21 to 31°C. Vitamin C was then shown to reduce the increase of body temperature during stress up to 35°C (Ahmad *et al.*, 1967), to reduce mortality associated with elevated ambient temperatures (Pardue *et al.*, 1985) and to improve immunoresistance (Pauling, 1976). Involvement of vitamin C in egg production during heat stress was also found. Njoku and Nwazota (1989) found that the inclusion of ascorbic acid in the diet improved egg production, food intake and food utilisation and decreased the cost of feed per kg of egg. The addition of 400 mg ascorbic acid per kg diet gave the most efficient performance.

Liposoluble vitamins requirements were also found to be affected by temperature. A threefold increase in vitamin A requirement was found in breeder hens at 38°C compared with those at normal room temperature (Scott, 1976). This increase was mainly attributed to a lower absorption of vitamin A during heat stress. Scott (1966) also found that the conversion of vitamin D_3 to the active form, which constitutes an important step for calcium metabolism, was affected during heat stress. Finally, vitamin E requirements have been shown to be increased during stress and particularly those that are related to high temperature to reduce heat stress-induced oxidative damage (Cheville, 1979).

2.3. Effect of vitamin E on heat-stressed poultry

2.3.1. Presentation of vitamin E

2.3.1.1. History

An excellent review of the history of vitamin E research has been provided by Scott (1980). The discovery of vitamin E dates back to 1922 when it was found that young rats reared on a diet consisting of purified protein, fat, carbohydrate, and containing adequate salts and vitamins were unable to produce offspring and that fertility was restored by the addition of lettuce in the diet (Evans and Bishop, 1922).

At first this substance was known as factor X, but Sure (1924) and Evans (1925) soon proposed the name vitamin E, since this was the next serial alphabetical designation. Vitamin E was isolated as α-tocopherol. The name tocopherol is derived from the Greek "tokos" meaning childbirth or offspring, the Greek "pherein" meaning to bring forth, and "ol" to designate an alcohol.

Throughout the 1920s, vitamin E was recognised only as a factor preventing testicular degeneration in rats. A few years later, Pappenheimer and Goettsch (1931) discovered that vitamin E was also required for prevention of encephalomalacia in chicks and of nutritional muscular dystrophy in rabbits and guinea pigs. By 1944, it was found that a multiplicity of clinical signs occur in animals suffering from vitamin E deficiency. In poultry, three distinct vitamin E deficiency diseases have been documented : exudative diathesis, encephalomalacia, and muscular dystrophy (section 2.3.2.2.). At the end of the 1940s, reproductive deficiency,

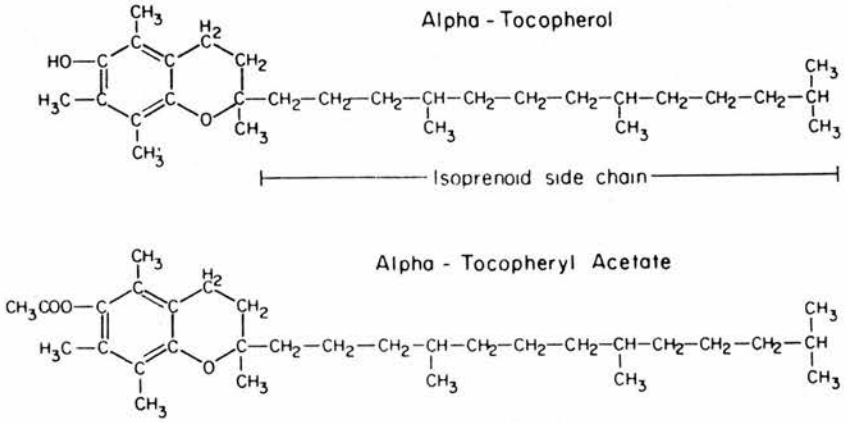
locomotor incoordination, hepatic necrosis, fibrinoid degeneration of blood vessel walls and muscular dystrophy were also reported in vitamin E-deficient pigs (Adamstone *et al.*, 1949; Obel, 1953).

While studying dietary liver necrosis of rats receiving a diet low in vitamin E, Schwartz *et al.* (1957) found that dried brewer's yeast, which contains no vitamin E, was as effective as vitamin E in preventing liver necrosis. Shortly after this discovery, selenium was found to be the active ingredient in brewer's yeast and able to replace vitamin E for prevention of exudative diathesis in poultry or tissue degeneration in swine. Much confusion existed because earlier discoveries had showed that synthetic antioxidants as well as sulphur amino acids were as effective as vitamin E for prevention of some vitamin E deficiency diseases. In recent years, considerable effort has been made to clarify the mechanisms of vitamin E, selenium, sulphur amino acids, and antioxidants in relation to vitamin E-responsive diseases.

2.3.1.2. Chemical structure and properties

Vitamin E activity in food derives from a series of compounds of plant origin, the tocopherols and the tocotrienols. Eight forms of vitamin E are found in nature : four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ). The structure of α-tocopherol and the commercially available α-tocopheryl acetate are presented in Figure 15. Differences between α, β, γ, and δ are due to the placement of the methyl groups on the chromanol ring. The difference between tocopherols and tocotrienols is due to unsaturation on the side chain in the latter.

Figure 15. Structure of α-tocopherol and α-tocopheryl acetate. From McDowell (1989).



The *dl* α-tocopheryl acetate, a racemic mixture of equal parts of dextro- and laevorotatory isomers, is accepted as the International Standard (1 mg = 1 international unit). Synthetic free tocopherol, *dl*-α-tocopherol, has a potency of 1.1 IU/mg. Activity of naturally occurring α-tocopherol, *d*-α-tocopherol, is 1.49 IU/mg, and of its acetate, 1.36 IU/mg. α-Tocopherol is a yellow oil that is insoluble in water but soluble in oils, fats and fat solvents. Tocopherols are extremely resistant to heat but readily oxidised. Natural vitamin E is subject to destruction by oxidation, which is accelerated by heat, moisture, rancid fat, and certain trace minerals. α-Tocopherol is an excellent natural antioxidant that protects carotene and other oxidisable materials in the body. However, in the process of acting as an antioxidant, it is inactivated but can be regenerated by other antioxidants (section 2.3.3.1.). Since esterification of the vitamin improves its stability, commercial supplements usually contain *d*-α-tocopheryl acetate or *dl*-α-tocopheryl acetate (section 2.3.2.2.).

2.3.1.3. Natural sources and procedures for analysis

Natural sources of vitamin E include vegetable oils, cereal products containing these oils, particularly the germs, eggs, liver, legumes, and, in general, green plants (Table 7). Other animal by-products supply only small amounts, and milk and dairy products are poor sources. As the stability of all naturally occurring tocopherols is poor, substantial losses of vitamin E activity can therefore occur, noticeably, after storage or processing manipulations.

Table 7. α-Tocopherol content of feeds (ppm). From Bauernfeind (1980) and Ullrey (1981).

Source	Mean*	Range
Alfalfa meal, dehydrated 17% protein	73	28 - 121
Barley, whole	36	22 - 43
Beef, meat	6	5 - 8
Brewer's grain, dried	27	17 - 48
Butter	24	10 - 33
Chicken, meat	3	2 - 4
Corn, whole	20	11 - 35
Eggs	11	8 - 12
Fat, animal	8	2 - 16
Fish meal, herring	17	8 - 31
Lard	12	2 - 30
Meat and bone meal	1	1 - 2
Oats, whole	20	18 - 24
Rice, bran	61	34 - 87
Wheat, whole	11	3 - 15

* To obtain an overall estimate of the content of total tocopherol, multiply value by 1.2 to account for the other tocopherols present.

Although biological methods have been used to compare and determine the potency of the different tocopherols (Table 8), many chemical assays have also been used for the determination of tocopherols in foodstuffs and animal tissues. Early methods were based on separation of the tocopherols by column, paper, or thin-layer chromatography, followed by a colorimetric reaction. More modern methods use high-pressure liquid chromatography (HPLC) (McMurray and Blanchflower, 1979) and involve three main steps, extraction, saponification, and chromatography.

Table 8. Relative biopotency of vitamin E forms. From Machlin (1984).

Name	Structure	Fetal resorption (rat) (%)	Hemolysis (Rat) (%)	Muscle dystrophy (chicken) (%)
α-tocopherol	5,7,8-Trimethyl tocol	100	100	100
β-tocopherol	5,8-Dimethyl tocol	25 - 40	15 - 27	12
γ-tocopherol	7,8-Dimethyl tocol	1 - 11	3 - 20	5
δ-tocopherol	8-Methyl tocol	1	0.3 - 2	-
α-tocotrienol	5,7,8-Trimethyl tocotrienol	29	17 - 25	-
β-tocotrienol	5,8-Dimethyl tocotrienol	5	1 - 5	-

Because α-tocopherol is the most active form of vitamin E and because the total activity contributed by other vitamin E forms is quite low, only the α-tocopherol concentration is generally is reported in foodstuffs. The total vitamin E activity can be approximated by multiplying the value in milligrams of α-tocopherol by 1.2 to account for other tocopherols that are present.

2.3.1.4. Metabolism

Vitamin E absorption is related to fat digestion and is facilitated by bile and pancreatic lipase (Ullrey, 1981) and is maximal in the median portion of the small intestine (no vitamin E is absorbed in the large intestine). Whether present as free alcohol or as esters, most vitamin E is absorbed as the alcohol. Esters are largely hydrolysed in the gut wall, and, after associating with portomicrons, the free alcohol enters the intestinal lacteals before transfer to the general circulation. Once in the circulation, vitamin E is transported via the portal vein directly to the liver. Balance studies indicated that the absorption, or at least the retention, of vitamin E is quite low and that only 20 to 40% of orally ingested tocopherol and/or its esters are absorbed

(Gallo-Torres, 1980). A major excretion route of absorbed vitamin E is represented by the bile, in which tocopherol appears mostly in the free form but also in the oxidised form.

The efficiency of absorption is enhanced by simultaneous digestion and absorption of dietary lipids and particularly medium-chain triglycerides, whereas polyunsaturated fatty acids are inhibitory (Machlin, 1984). As they compete with vitamin E, vitamin A and carotenoids were also found to reduce vitamin E absorption (Combs, 1976).

As the various tocopherols and tocotrienols are absorbed in the same general order of magnitude as their biological potencies, most of the vitamin E activity within plasma and other animal tissues is due to α-tocopherol (Ullrey, 1981). In the plasma, 40 to 60% of vitamin E is attached mainly to the low density lipoproteins (LDL) in the globulin fraction, and 35% is attached to the high density lipoproteins (HDL). Within the body, vitamin E is stored in all tissues, with highest concentrations in the liver and adipose tissues. Dietary vitamin E supplementation has been shown to elevate vitamin E levels in chick tissues, such as liver, heart, skeletal muscle and plasma ($P<0.05$) (Woodall *et al.*, 1996). Upon withdrawal of vitamin E from the diet, its rate of depletion varies considerably, being relatively rapid in plasma and liver, slower from skeletal and heart muscle, and very slow from adipose tissue (Machlin and Gabriel, 1982).

2.3.1.5. Physiological roles

2.3.1.5.1. Biological antioxidant

The most important function of vitamin E is its role as an antioxidant in cell and organelle membranes. In this capacity, it prevents oxidation of unsaturated lipid materials by inactivating free radicals, thus protecting fats within cell membrane from breaking down. This role of vitamin E, which assures a continuous protection within the cell, is particularly important in animals subjected to stress, is documented in detail in section 2.3.3.2.

2.3.1.5.2. Membrane structure and prostaglandin synthesis

Vitamin E is most concentrated in cell fractions rich in membrane, such as the mitochondria and microsomes. Recent reports have demonstrated the existence of proteins in the cytosol that

specifically bind α-tocopherol. The cytosolic binding proteins apparently facilitate the transport of tocopherol into the mitochondrial (Mowri *et al.*, 1981) and microsomal membranes (Murphy and Mavis, 1981). Although γ-tocopherol is absorbed and deposited in tissues readily, it is retained only to a limited degree compared with α-tocopherol. Thus, the difference of one methyl group on the chromonal ring (section 2.3.1.2.) has a profound effect on tissue retention. Lucy (1978) has proposed that the phytyl side chain may interact with the fatty acyl chain of polyunsaturated phospholipids, particularly those derived from arachidonate. Alterations of the side chain drastically alter biological activity (Kasparek, 1980). Thus, the structure of both the ring and side chain may be important in determining the specific locus of the vitamin in the cell membrane.

α-Tocopherol also participates in the formation of structural components of biological membranes, thus exerting a unique influence on architecture of membrane phospholipids (Lucy and Diplock, 1972). It was reported that α-tocopherol stimulates the incorporation of ^{14}C from linoleic acid into arachidonic acid in fibroblast phospholipids. Also, it was found that α-tocopherol exerts a pronounced stimulatory influence on formation of prostaglandin E from arachidonic acid, while a synthetic antioxidant had no effect.

2.3.1.5.3. Blood clotting

Vitamin E is an inhibitor of platelet aggregation, and may participate in this mechanism by inhibiting peroxidation of arachidonic acid. The latter, which is found in membranes, is converted by cyclic oxidation into prostaglandins, which are thought to stimulate platelet aggregation (Panganamala and Cornwell, 1982). Vitamin E counters this reaction and is well-positioned to do so due to its localisation within cellular membranes. Among antioxidants characterised to date, this role appears unique to vitamin E.

2.3.1.5.4. Disease resistance

The mechanisms whereby cells immunologically kill invading bacteria has been shown to involve vitamin E and selenium. Both of these compounds help leucocytes and macrophages to survive the toxic products that are produced in order to effectively kill ingested bacteria (Badwey and Karnovsky, 1980). Supplementation of vitamin E above recommended levels has been shown to improve resistance to microbial infections (Nockels, 1979). This improved

resistance apparently occur through an improved immune function (Lawrence *et al.*, 1985) and greater production of antibodies, particularly IgG (Tengerdy *et al.*, 1973). In agreement with these findings, it has been reported that administration of vitamin E increases the phagocytotic activity of the reticulo-endothelial system or RES (Heinzerling *et al.*, 1974) with increase in the weight of the spleen and in the numbers of antibody producing cells within this organ (Tengerdy *et al.*, 1973). Vitamin E appears to stimulate the defence mechanisms by increasing ubiquinone synthesis, because ubiquinones increase the phagocytoyic activity of the RES (Heinzerling *et al.*, 1974). Protection against *E. coli* infection has been demonstrated in six week old chicks fed 300 mg vitamin E per kg as antibody production was increased and mortality was reduced from 40% to 5% (Tengerdy and Nockels, 1975).

2.3.1.5.5. Other functions

There is limited evidence that vitamin E is involved in biological oxidation-reduction reactions (Anonymous, 1972). It may act as a cofactor in the cytochrome reductase portion of the nicotinamide-adenine dinucleotide (NAD) oxidase and the succinate oxidase systems. Restoration of the specific activity of cytochrome C reductase by vitamin E as been shown and this vitamin alone can reactivate this enzyme system following its inactivation by isolation and ageing or freezing and thawing. Vitamin E also appears to regulate the biosynthesis of deoxyribonucleic acid (DNA) within cells and to participate in the synthesis of the heme and of some heme proteins (cytochromes, hemoglobin).

Additional functions of vitamin E have been reported. Vitamin E provides protection against toxicity of heavy metals, such as silver, arsenic and lead (Whanger, 1981). It assures normal phosphorylation reactions, especially of high-energy phosphate compounds such as creatine phosphate and adenosine triphosphate (Scott *et al.*, 1982), participates in the synthesis of ascorbic acid and ubiquinone and affects sulphur amino acid metabolism. Vitamin E has also been reported to have a role in vitamin B_{12} metabolism (Pappu *et al.*, 1978).

2.3.2. Vitamin E in poultry nutrition

2.3.2.1. Requirements

Estimated vitamin E requirements in poultry, selected animals and human are presented in Table 9. After reviewing the literature, Scott (1980), concluded that the minimum vitamin E

requirement in poultry generally varies between 10 and 20 mg per kg of diet. However, the requirements are exceedingly difficult to determine because of the interrelationships with other dietary factors. They may be increased with increasing levels of poly unsaturated fatty acids (PUFA) which increase the need for antioxidant protection in the body, oxidising agents, trace minerals, vitamin A and carotenoids (section 2.3.1.4.), and decreased with increasing levels of fat-soluble antioxidants, sulphur amino acids, and selenium (section 2.3.1.5. and 2.3.2.2.). Determination of vitamin E requirements is also further complicated by the fact that the body has a fairly large ability to store vitamin E and selenium (Hidiroglou *et al.*, 1992).

Table 9. Vitamin E requirements for poultry and other animals (as proportion of diet) and humans (daily intake).

Animal	Requirement	Reference
Growing chicken, 0 - 6 weeks	10 IU/kg	NRC (1984)
Growing chicken, 6 - 20 weeks	5 IU/kg	NRC (1984)
Laying hen	5 IU/kg	NRC (1984)
Broiler, 0 - 8 weeks	10 IU/kg	NRC (1984)
Growing turkey, 0 - 8 weeks	12 IU/kg	NRC (1984)
Growing turkey, 8 - 24 weeks	10 IU/kg	NRC (1984)
Breeding turkey	25 IU/kg	NRC (1984)
Dairy cattle	300 IU/kg	NRC (1978)
Sheep	15 - 20 IU/kg	NRC (1985)
Swine	11 - 22 IU/kg	NRC (1988)
Rabbit	40 IU/kg	NRC (1977)
Human, adult	8 - 13 mg/day	RDA (1980)

2.3.2.2. Supplementation

Methods of providing supplemental vitamin E are (1) as part of a concentrate or liquid supplement, (2) included with a free-choice mineral mixture, (3) as an injectable product, and (4) in drinking water preparations. Although the most active form of natural vitamin E found in feed ingredients is *d*-α-tocopherol, the principal commercially available forms of vitamin E used in the food are acetate and hydrogen succinate esters of RRR α-tocopherol and the acetate of all-rac-α-tocopherol. During commercial synthesis, *dl*-α-tocopherol is esterified to the acetate for increased stability and resistance to oxidation. *dl*-α-Tocopherol acetate does not act as an antioxidant in the feed and only has antioxidant activity after it is hydrolysed in the intestine and free *dl*-α-tocopherol is released and absorbed (Mc Dowell, 1989).

2.3.2.3. Deficiency

Vitamin E shows one of the widest ranges of deficiency signs of all vitamins. Although vitamin E deficiency signs differ among species and even within the same species, Blaxter (1962) reported that muscular dystrophy, which consists of degeneraration of both skeletal and cardiac muscle fibres, seemed to be the one syndrome commonly encountered in vitamin E deficiency in all species.

In chickens, vitamin E deficiency can result in three main conditions : exudative diathesis, encephalomalacia and muscular dystrophy. Exudative diathesis is responsible for subcutaneous edema produced by a marked increase in capillary permeability and, in severe cases, blackening of affected parts, apathy, and inappetance. It can be prevented by administration of vitamin E or selenium. Encephalomalacia generally affects chicks from 2 to 6 weeks of age and results from haemorrhages and oedema within the cerebellum. At least one important function of vitamin E is to interrupt the production of free radicals at the initial stage of encephalomalacia. The quantitative need for vitamin E for this function depends on the amount of linoleic acid in the diet. Selenium is ineffective in preventing encephalomalacia, while synthetic antioxidants are highly effective. Muscular dystrophy, especially of breast muscle, occurs when vitamin E deficiency is accompanied by sulphur amino acid deficiency and can be prevented by administration of selenium and vitamin E.

2.3.2.4. Toxicity

Hypervitaminosis E studies in chicks indicate maximum tolerable levels in the range of 1,000 to 2,000 IU per kg diet (NRC, 1987). Doses of 4,000 IU per kg diet gave decreased pigmentation in beaks, feet and shanks, and liver as a percent of body weight was significantly increased (Nockels *et al.*, 1976). Doses of 5,000 IU per kg diet caused reduced packed-cell volumes (NRC, 1981) and exacerbated the coagulation defect associated with a vitamin K deficiency in chicks (Corrigan, 1982). Feeding at least 8,000 IU per kg diet significantly reduced chick body weight and resulted in a waxy appearance of feathers.

2.3.3. Vitamin E and oxidative stress

As mentioned earlier (section 2.2.5.), when an organism is exposed to heat stress, the formation of free radicals and ROS increases. If these radicals are not reduced by antioxidants, tissue damage can ensue. This condition, which results from an imbalance between pro-oxidants and antioxidants, is often referred to as oxidative stress (Sies, 1991). As a wide range of health problems have been associated with the generation of free radicals and ROS in humans, e.g. cancer, diabetes, atherosclerosis, cystic fibrosis, HIV infection and many others (Halliwell, 1989), and also in poultry, e.g. ascites or pulmonary hypertension syndrome (Bottje *et al.*, 1995), considerable work has been devoted to understand the mechanisms involved in the antioxidant defence against oxidative stress.

2.3.3.1. Antioxidant defences

The antioxidant systems responsible for protecting cells from the actions of free radicals and ROS are diverse, intertwined and many layered. There are two classes of antioxidants whose actions are relevant *in vivo* (Halliwell, 1995).

A first class of antioxidants is synthesised and controlled endogenously. These antioxidants, classified as preventative, include enzymes, proteins and certain by-products of metabolism. Enzymatic antioxidants include superoxide dismutases (Mn SOD, Cu SOD and Cu/Zn SOD), glutathione (GSH) recycling system (GSH peroxidase or GSHPx, GSH reductase or GSHRd) and catalase. Since essential minerals including Se, Cu, Mn, Zn and Fe are components of these protective systems, mineral deficiencies might hamper the body's enzymatic defences. Except for Cu SOD, all of these antioxidants are located within the cell in various organelles, such as mitochondria where most of the intracellular free radical production occurs (Chance *et al.*, 1979), but also in peroxisomes, nucleus or cytosol. Their functions are varied, SODs catalyse the dismutation of $O_2^{\cdot-}$ to H_2O_2, GSHRd reduces low molecular weight disulphides (GSSG-GSH) using NAD(P)H, GSHPx reduces H_2O_2 and other hydroperoxides during "normal" metabolism, and catalase reduces H_2O_2 mainly during diseases or stress states (Jones *et al.*, 1981). The interaction between these enzymes can be represented by the following relation (Eq. 4) :

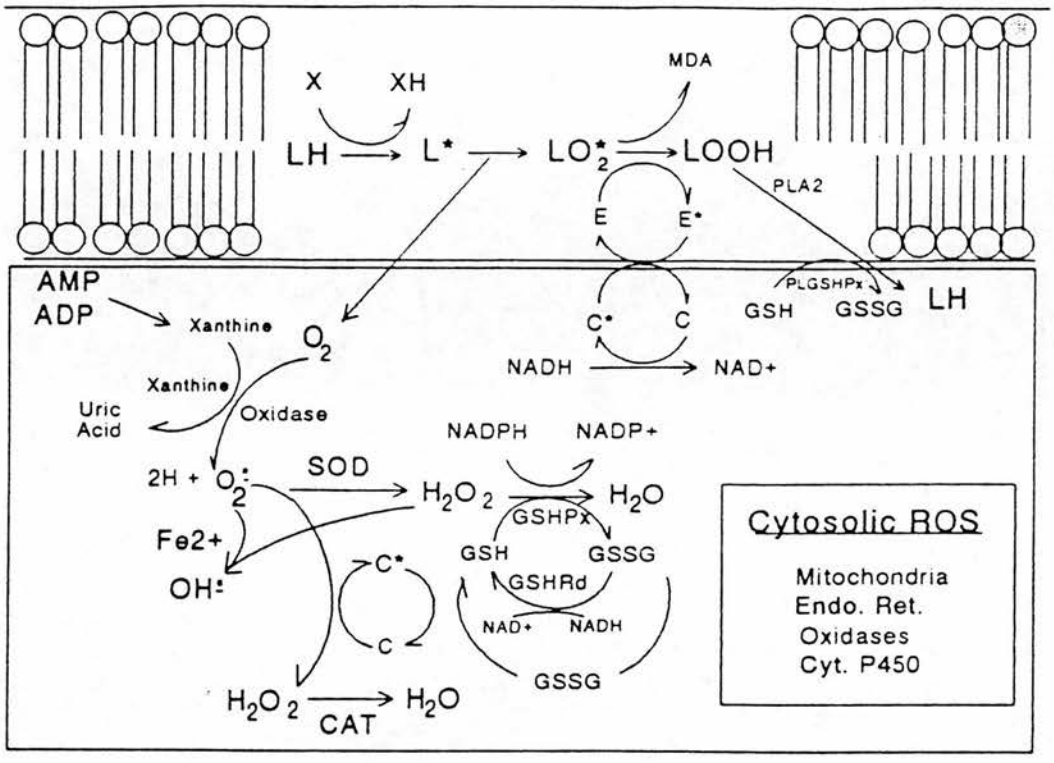
$$\text{(Eq. 4)} \qquad O_2^{\cdot-} \xrightarrow{\text{SOD}} H_2O_2 \xrightarrow[\text{CAT}]{\text{GSHP}_x} H_2O$$

Among the proteins and other endogenously synthesised antioxidants are GSH, uric acid, cysteine and glucose. GSH is mainly found within the cell, interacts directly with $O_2^{\cdot-}$, $OH^{\cdot}$ and hydroperoxides and serves as substrate for GSH recycling enzymes. Uric acid, cysteine and glucose are widely distributed and demonstrate functions which vary from binding transition metals, interaction with $O_2^{\cdot-}$, $OH^{\cdot}$ and peroxyl radicals to prevention of oxidation of ascorbic acid (Ames *et al.*, 1981).

A second class of antioxidants is represented by those which are provided by dietary means, classified as chain-breaking (for reviews see Frei, 1994; Kehrer, 1993). This category includes lipid-soluble, chain-breaking agents such as vitamin E, ubiquinone, retinoic acid and β-carotene and water-soluble substances such as vitamin C and GSH. Among the vitamins, vitamin E, vitamin C and β-carotene are potent, direct-acting antioxidants whereas vitamin A is a less active antioxidant. With the exception of vitamin C, they are located in membranes and extracellular fluid where they convert or scavenge $O_2^{\cdot-}$, $OH^{\cdot}$ and lipid peroxyl radicals to less reactive compounds and protect against lipid peroxidation. Vitamin C, which is widely distributed in intra- and extra-cellular fluid, interacts directly with $OH^{\cdot}$, reduces $O_2^{\cdot-}$ to H_2O_2, neutralises ROS (Combs, 1992) and regenerates vitamin E from the tocopherol radical form (Witting and Horwitt, 1964). Excess of vitamin C can, however, have pro-oxidant consequences by reducing Fe^{3+} to Fe^{2+} which can generate free radicals via the Fenton reaction or by interaction with Cu^{2+} (section 2.2.5.). β-Carotene can also act as a pro-oxidant at higher dietary concentrations.

The cooperative interaction between cellular antioxidants protecting against free-radical mediated injury is summarised in Figure 16. Depicted at the top of the figure is a lipid bilayer where abstraction of an electron from a lipid (LH) by a free radical (X) generates a lipid peroxyl radical ($L^{\cdot}$). With the addition of oxygen, a lipid peroxide ($LOO^{\cdot}$) is formed with the concomitant release of MDA. Vitamin E reduces the lipid hydroperoxide to a lipid alcohol (LOOH) that is released from the membrane by phospholipase A_2 (PLA2). Once released, the lipid hydroperoxide can be converted to an alcohol by GSHPx. In reducing the lipid hydroperoxide, vitamin E is converted to a tocopherol radical ($E^{\cdot}$). Movement of ascorbic acid in close proximity to the lipid-cytosol interface allows the regeneration of reduced vitamin E. The dehydroascorbate radical can be reduced back to ascorbate through reducing equivalents provided by NADH.

Figure 16. Summary of cellular antioxidant protective mechanisms (From Bottje and Wideman, 1995).



Cytosolic components of the antioxidant protective mechanisms are also represented in Figure 16. The conversion of xanthines and hypoxanthines by xanthine oxidase under hypoxic conditions produces uric acid and $O_2^{\cdot-}$. Superoxide is converted to H_2O_2 by SOD and then reduced to water by either GSHPx or catalase. In the presence of transition metals, $O_2^{\cdot-}$ and H_2O_2 are converted to the highly reactive $OH^{\cdot}$ which highlights the important antioxidant role that the concerted activities of SOD, GSHPx and catalase have in metabolising free radicals and ROS. The oxidised GSH (GSSG) formed during this process is reduced to GSH by GSHRd using reducing equivalents from NADPH produced by the pentose-phosphate shunt. There is also some evidence that GSSG may be reduced to GSH by vitamin C *in vitro*.

There is a close working relationship between vitamin E and selenium within tissues. Selenium has a sparing effect on vitamin E and delays onset of deficiency syndromes. Likewise, vitamin E partially protect against or delay onset of several forms of selenium deficiency syndromes (McDowell, 1989). To explain this interaction, McCay and King (1980) proposed that $O_2^{\cdot-}$ reacts with hydrogen ions to produce H_2O_2 which is then distributed in both the aqueous and membrane phases of the cell. Selenium, as part of GSHPx, destroys H_2O_2 in the aqueous

phase, thus shifting most of H_2O_2 into the membrane. Any H_2O_2 remaining in the membrane may react with $O_2^{\cdot-}$ to form $OH^{\cdot}$, which can react with tocopherol localised in the membrane. If insufficient tocopherol is available to trap $OH^{\cdot}$, this extremely reactive radical may initiate peroxidation of PUFA in the membrane. This hypothesis accounts for many experimental observations, including the role of selenium (as part of GSHPx), in alleviating some of the symptoms of vitamin E deficiency.

Finally the regeneration of the antioxidant form of vitamin E by vitamin C and coenzyme ubiquinol has been shown to explain the involvement of the last two in the protection of membranes and lipoprotein particles from lipid peroxidation (Beyer, 1994).

2.3.3.2. Vitamin E and lipid peroxidation

Vitamin E has been recognised for some time as the major, if not the only, lipid-soluble chain breaking antioxidant in blood plasma, i.e. factor which neutralises peroxyl and alkoxyl radicals and prevents further peroxidative damage of membranes (Tappel, 1962; Burton *et al.*, 1983). Due to its high lipid solubility, it is located in plasma and organelle membranes, e.g. mitochondria and endoplasmic reticulum.

Molenaar *et al.* (1980) proposed that the chromanol ring of tocopherol is located at the polar surface of membranes and that the phytol side chain interacts with PUFA of the phospholipids (Lucy, 1978) in the nonpolar interior of the membrane. The progressive peroxidation of PUFAs which occurs in the presence of free radicals, ROS or oxygen can therefore be alleviated, at least in part, by vitamin E. It is also possible that tocopherol is an effective membrane radical scavenger because it is able to move very rapidly through the nonpolar portion of the membrane and reach membrane-bound enzymes, such as NADPH oxidase, that generate free radicals. It was further proposed that tocopherol donates the hydrogen from the phenolic hydroxyl group present in the 6-hydroxychromane ring to the chain-propagating lipid peroxyl and alkoxyl radical intermediates of lipid peroxidation thus terminating the chain reaction (Ruckebrush *et al.*, 1991). There is also some evidence for an alternative pathway whereby vitamin E also exerts a protective effect by converting the peroxyl radical into the parent unoxidised lipid molecule with the concomitant formation of superoxide. This pathway has been demonstrated in a liposomal model membrane system (Dmitriev *et al.*, 1994).

Using heterogenous mixtures of vesicles derived from both endoplasmic reticulum and plasma

membranes, or microsomes (Halliwell and Gutteridge, 1989) and artificial lipid structures made by shaking or sonication of phospholipids in aqueous suspension, or liposomes (Chatterjee and Agarwal, 1988; Kagan *et al.* (1990) demonstrated the *in vitro* inhibition of lipid peroxidation by α-tocopherol. The membrane antioxidant action of α-tocopherol enables it to protect cultured human endothelial cells against linoleic acid hydroperoxide-induced cytotoxicity resulting from hydroperoxide-induced lipid peroxidation (Kaneko *et al.*, 1994).

Recent studies have suggested that vitamin E may also play a role against cellular damage by processes not directly related to its ability to inhibit free-radical reactions. Some authors reported protective effects of vitamin E on the skeletal muscle which appeared to be related to its ability to stabilise or change the fluidity of the membrane rather than to its antioxidant effect (Phoenix *et al.*, 1991; Page *et al.*, 1993).

These effects can partly explain why vitamin E was found to improve meat quality and shelf life. Studies on the effect of providing different doses of vitamin E to chickens aged 0 to 7 weeks showed that the oxidative stability (measured as TBA values) of drumstick meat stored at -18°C for 20 days was significantly ($P<0.01$) negatively correlated with the amount of vitamin E consumed during the experiment (Bartov and Frigg, 1992).

2.3.4. Vitamin E and heat-stress in poultry

2.3.4.1. Muscle damage

Damage to skeletal muscle during or following transportation or exposure to stressful conditions is a well-recognised phenomenon in mammals. Interest in the possibility that oxygen-centred free radicals could be involved in the damage which occurs following exercise was prompted by the suggestion that the myopathy observed in vitamin E-deficient cattle was precipitated by unaccustomed exercise (Allen *et al.*, 1975). Vitamin E is known to exert its major effect in the body by detoxification of lipid-soluble free radicals and the inference was therefore drawn that, in the rat, free radical production may be increased by exercise (Brady *et al.*, 1979). There is a large increase in oxygen utilisation by mitochondria during exercise and it was suggested that this may increase the oxidative stress to the tissues in exercising subjects. Studies both *in vitro* and *in vivo* have demonstrated that a low muscle vitamin E content potentates the damage to skeletal muscle which occurs for a given stress. However, addition of an excess of vitamin E to the medium surrounding isolated normal muscle did not modify the

response to a damaging stimulation (Jackson, 1987).

Experiments carried out on broilers showed that an exposure to an acute stress at 35°C for two hours resulted in a plasma creatine kinase rise which is due to the release of it from muscle cells in response to an intracellular influx of calcium in these cells. In broilers fed 300 mg vitamin E per kg during 8 weeks and exposed to the same type of stress, the severity of the stress-induced plasma creatine kinase rise was reduced ($P<0.05$) compared to non-supplemented birds showing that vitamin E ameliorates some of the metabolic consequences of acute heat stress in broilers (Whitehead *et al.*, unpublished).

2.3.4.2. Pulmonary hypertension syndrome

Excessive free radical generation during inflammation can cause damage to surrounding tissues, particularly to the lung. Free radicals and ROS generated in the lower respiratory tract by phagocytes involved in local inflammatory reactions, have been recognised as important factors in the aetiology of various lung diseases, such as ascites which is a consequence of pulmonary hypertension syndrome (PHS) (Ryrfeldt *et al.*, 1993; Cantin and Crystal, 1985).

Sudden death syndrome (SDS), in which birds die of sudden cardiovascular failure, has many characteristics similar to PHS and may simply be an extreme manifestation of PHS. Squires and Summers (1993) hypothesised that the decrease in mortality due to sudden death syndrome observed when birds were fed animal fat might result from a lowered intake of polyunsaturated fatty acids which are more susceptible to lipid peroxidation. It should also be pointed out that under certain conditions saturated fatty acids may facilitate, and unsaturated fatty acids diminish, the absorption of lipid soluble antioxidant vitamins (Hollander, 1981). Thus, the higher incidence of sudden death in birds fed higher levels of unsaturated fats may be due to increased susceptibility to lipid peroxidation compounded by decreased absorption of vitamin E and A needed to protect membrane from peroxidation. Disruption of the equilibrium between thromboxane (vasoconstrictor) and prostacyclin (vasodilator) which occurs when lipid peroxide concentration increases or when antioxidant levels are inadequate might also contribute to the development of pulmonary hypertension syndrome in broilers (McNamara *et al.*, 1985). Furthermore, H_2O_2 and other lipid hydroperoxides were found to cause vaso- and broncho-constriction via synthesis and release of arachidonic acid metabolites (Gurtner *et al.*, 1987).

Bottje and Wideman (1995) demonstrated lower lung tocopherol levels in birds which had fulminant PHS than in normal controls. In birds which received a 3-week vitamin E implant

and aged of 3 or 5 weeks, vitamin E concentrations in the lung were 199% and 168%, respectively, of control values (Bottje *et al.*, 1995). As the lung, specifically the pulmonary vasculature, seems to be the primary organ involved in the development of PHS, and thus particularly vulnerable to oxidative stress, the elevation of vitamin E combined with lower PHS mortality suggests that pulmonary antioxidant protection may be particularly important in lowering PHS mortality. Enkvetchakul *et al.* (1993) also demonstrated a decrease in the concentration of vitamin E in the lungs of birds suffering from ascites than in control birds.

2.3.4.3. Liver damage

In the presence of free radicals, ROS or oxygen, PUFAs undergo progressive peroxidation (section 2.3.3.2). This chain-reaction was found affect the liver and to generate hepatic necrosis (Ruckebrush *et al.*, 1991). Dietary supplementation with vitamin E (50 mg per kg) was shown to reduce heme protein oxidation and lipid peroxidation initiated by incubation of liver slices with ferrous sulphate (Pellett *et al.*, 1994). Ethanol-induced oxidative stress can be partly prevented by vitamin E supplementation. Woodall *et al.* (1996) also demonstrated that feeding 100 mg vitamin E/kg to chicks resulted in a reduced susceptibility to oxidative stress in liver. Spurlock and Savage (1993) demonstrated that providing 200 mg vitamin E per kg to Japanese Quail reduced fatty liver haemorrhagic syndrome and attributed this to the antioxidant or structural role of vitamin E.

2.3.4.4. Egg production

An experiment carried out by Utomo *et al.* (1994) demonstrated the beneficial effect of high doses of vitamin E in heat-stressed laying hens. As mentioned earlier (section 2.2.6.), egg production and egg weight are reduced during heat stress. However in their experiment, Utomo *et al.* (1994) found that supplementing birds with 500 mg vitamin E per kg for five weeks (2 weeks at 22°C, one week at 35°C and two weeks at 22°C) resulted in an increase in egg production and egg weight during and after heat stress compared to those of birds fed a control diet (10 mg vitamin E per kg). Increases of 0.18 eggs per hen day and 3.5 g were obtained in egg production and egg weight respectively during the week of heat stress and increases of 0.20 eggs per hen per day and 4.0 g were obtained for the same respective criteria during the 2-week post-heat stress period.

In the same experiment, plasma concentrations of zinc (measured as an indicator vitellogenin)

and of triglycerides, were also found to be improved by the vitamin E treatment with increases of respectively 45% and 45% during heat stress and 50% and 40% after heat stress. These results suggested that vitamin E may have a role in protecting hens from the effects of heat stress and in maintaining egg production. As cellular damage is reduced by the anti-oxidant effect of vitamin E during exposure to stressful conditions, egg yolk precursor synthesis may have been increased in the liver and/or incorporation of these precursors may have been increased into ovarian follicles. Alternatively, vitamin E may have improved hormonal status and increased the circulating concentration of steroid hormones during heat stress.

The objective of the research described in this thesis were to study the nutritional effects of vitamin E on laying hens under heat stress and to try to establish the mechanism(s) involved.

Chapter 3

General materials and methods

This chapter documents the materials and methods common to all the experiments. More specific methods are described in individual chapters.

3.1. Data collection

3.1.1. Egg production

Every morning, all the eggs laid within the previous 24-hours were recorded and weighed. In large scale experiments, weight of eggs were measured to 1 g using a balance (Sartorius) connected to a nanocomputer (Epson). In other experiments, weight of eggs was measured to 0.01 g.

3.1.2. Food consumption

Food consumption was measured on an individual basis. In the large scale experiments, food consumption was determined weekly by measuring the weight difference in bucket of food allocated to each bird. This method was also used in other experiments where the food consumption was determined every 3 or 4 days.

3.1.3. Body weight

The body weight of each bird was measured at the beginning of the experiment and at the end of every four week period in the large scale experiments. Weights were recorded to 1 g. In the other experiments, individual body weight was determined at the beginning of the experiment and at the end of every temperature treatment (or period) to the nearest 10 g.

3.2. Blood analyses

In all the experiments, blood samples (approximately 3 ml) were obtained from birds by venepuncture of the brachial vein, using heparinised syringes and transferred to heparinised tubes (heparin in physiological saline 50 units/ml, Evans Medical Ltd). Plasma was collected after centrifugation of blood at 1,500 g for 10 minutes (name of centrifuge) and stored at -20°C until required for analysis.

Some plasma characteristics were analysed in most of the experiments. These included determination of the plasma concentrations of proteins, total zinc, vitellogenin (as zinc), very low density lipoproteins (as triglycerides). Each assay was modified and validated for use with avian plasma and for reading with the aid of an automated plate reading spectrophotometer (Titer-tek 2, Autoflow Laboratories UK).

3.2.1. Protein

Protein concentration in the plasma was determined by spectrophotometry using the Coomassie brilliant blue G-250 (55% phosphoric acid, 15% methanol) Bradford dye-binding procedure (Bio-Rad Laboratories, UK). This assay is based on the colour change of Coomassie brilliant blue G-250 dye in response to various concentrations of protein. The dye binds primarily to basic (especially arginine) and aromatic amino acid residues. The assay is useful for measuring proteins and polypeptides with M_r greater than 3 to 5 kDa, depending on the charged groups. γ-globulin (2 mg/ml) was used as a protein standard and diluted 2, 4, 8 and 16-fold in order to give standard concentrations of 2, 1, 0.5, 0.25 and 0.125 mg/ml. To fall within this range of concentrations (i.e. lower than 2 mg/ml), plasma samples had to be diluted 50-fold and then 5 µl of each was added into a well of a microplate. Bio-Rad reagent concentrate was diluted 5-fold and 250 µl was added to each sample. The protein concentration was measured as absorbance shift in Coomassie Brilliant blue solution at 595 nm 15 minutes following protein binding (Bradford, 1976).

3.2.2. Total zinc

Total zinc was determined by spectrophotometry using a specific chelating agent, the sodium salt of 2-(5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino)-phenol or 5-Br-PAPS (Wako Chemicals GmbH). First, all samples including blanks and standards were deproteinised by addition of trichloroacetic acid to leave the zinc in solution. An aliquot of the supernatant was

then mixed with 5-Br-PAPS, which in the presence of zinc forms a purple-red chelate. The absorption of the chelate was measured at 560 nm and was directly proportional to the amount of zinc in the sample. The result is linear up to 153 μmol/l. Although the colour reagents employed can also be used for the determination of iron, copper, cobalt, and nickel, reaction with these other metals is suppressed by masking agents that are contained in the assay kit. 5-Br-PAPS chelates zinc with a 2:1 molar stoichiometry. Zinc solution of concentration 2 μg/ml (30.6 μmol/l) of zinc was used as a standard and diluted 2, 4 and 8-fold in order to give standard concentrations of 2, 1, 0.5 and 0.25 μg/ml. Aliquots (100 μl) of standards, blank and samples were mixed with an equal volume of 7% trichloroacetic acid by vortexing and then centrifuged for 10 minutes at 2,500 g. 40 μl of the supernatant was then removed and added into a well of a microplate. Chromogen solution was added (200 μl) and the zinc concentration was measured as an absorbance shift after leaving the solution for 10 minutes at room temperature (Hiraki, 1973; Shiraishi, 1970).

3.2.3. Vitellogenin

Vitellogenin-bound zinc was used as an indicator of vitellogenin as described by Mitchell and Carlisle (1991). These authors showed that, in plasma, after treatment with dextran sulphate-magnesium chloride, the amount of zinc present in the pellet was equivalent to the amount of vitellogenin-bound zinc. A solution containing 0.9% NaCl, 2% dextran sulphate, 200 mM $MgCl_2$ and 10 mM Tris buffer, adjusted to pH 7.4 with HCl was added to each plasma sample to precipitate both vitellogenin and triglyceride-rich VLDL (Griffin and Mitchell, 1984) and the concentration of zinc present in the supernatant was then determined as described in section 3.2.2. The concentration of zinc present in the supernatant was then subtracted from the total zinc (see section 3.2.2.) to give the amount of zinc bound to vitellogenin.

3.2.4. Very Low Density Lipoprotein

The concentration of triglyceride was used as an indicator of the concentration of VLDL. Triglyceride was determined by spectrophotometric assay using an enzyme cocktail which first hydrolyses triglyceride into glycerol (catalysed by lipoprotein lipase) and then generates glycerol-3-phosphate in the presence of ATP (catalysed by glycerol kinase). The glycerol-3-phosphate is oxidised by glycerol-3-phosphate oxidase to yield dihydroxyacetone phosphate and hydrogen peroxide. The latter reacts with 4-aminoantipyrine and p-chlorophenol (catalysed by peroxidase) to give a red quinoneimine dye detectable at a wavelength of 505 nm. The linearity extends to

11.3 mmol/l. The plasma were diluted 3-fold with distilled H_2O to fall within range of standards and the results obtained multiplied by 3. Triglyceride was measured by means of a commercial kit (Triglyceride-N, Wako Chemicals GmbH) using tri-oleins as standard at a maximum concentration of 3 mg/ml. Halving dilutions were made to produce the other two standards (1.5 and 0.75 mg/ml). Standards, blank and samples were pipetted into wells of a microplate in a volume of 6 µl and 300 µl of colour reagent added. They were then read on a plate reader within 30 minutes of adding colour reagent.

3.3. Statistical analyses

3.3.1. Large scale experiments

The two large scale experiments carried out in this thesis were factorial experiments. Data from the first experiment (Experiment 1) were analysed using split plot analyses of variance between and within groups (blocks) of three adjacent hens with treatment effects of room, dietary level of vitamin E and the interaction between these two factors. Data from the second experiment (Experiment 2) were analysed using randomised blocks analyses of variance with treatment effects of vitamin E status (short or long supplementation duration), dietary level of vitamin E, the interaction between these two factors and an additional contrast of the control diet versus all other treatments. In both experiments, all data relating to egg production, food consumption and body weight were compared on a four-week basis using an analysis of variance (Anova) (Genstat 5 package, Lawes Agricultural Trust). The statistical significance of treatment effects were evaluated by Student's t test or the variance ratio test using the appropriate degrees of freedom and residual error variance provided by the analyses of variance. When regression equations were calculated, the type of regression used was indicated.

3.3.2. Other experiments

All egg production, food consumption and body weight data for each experimental period were compared using a Student t-test (Minitab statistical package). As mentioned earlier, when regression equations were calculated, the type of regression used was indicated.

Chapter 4

Effect of vitamin E on egg production in heat-stressed laying hens

In a previous study, Utomo *et al.* (1994) demonstrated that a high dose of vitamin E (500 mg of α-tocopherol per kg of diet) increased egg production of birds exposed to a period of heat stress (section 2.3.4.4.). After a week at 32°C, the number of eggs produced per hen per day was increased by 22% in birds fed this highly supplemented diet compared to that of birds fed a control diet (30 mg of α-tocopherol per kg diet). However, as this trial was performed on a small number of birds (12 birds in each group), the result was not statistically significant.

The objectives of the experiments described in this chapter were therefore to validate this result on a larger scale and to determine the optimum conditions for using vitamin E (dose and duration of supplementation) which lead to a significant improvement of egg production in heat stressed laying hens.

4.1. Experiment 1

The objective of this experiment was to investigate whether the dose used by Utomo *et al.* (1994), 500 mg of α-tocopherol per kg of diet, had any positive effect on egg production of laying hens exposed to a chronic period of heat stress of 4 weeks at 32°C which was applied to birds early or later in lay. To study the pattern of the response of egg production to vitamin E supplementation, an additional dose of vitamin E was also tested : 125 mg of α-tocopherol per kg of diet on the two groups of birds. Another objective was to determine the statistical significance of the results, therefore large numbers of birds were used : 50 birds per treatment instead of 12 in the experiment of Utomo *et al.* (1994).

4.1.1. Materials and methods

4.1.1.1. Selection of the birds and husbandry

Six hundred ISA Brown hens were housed at 16 weeks of age in individual cages in a climate room at 22°C for an adaptation period of four weeks. They had *ad libitum* access to pelleted pre-lay diets for which composition and characteristics are described in the Annexe (Table A1). At 16 and 20 weeks of age, the birds were individually weighed, the live-weight gain between weeks 16 and 20, the body weight and the pelvic gap at week 20 were used as criteria to select 300 birds in good laying condition. These birds were then housed in individual cages in three tier battery units in two temperature controlled rooms. The light period was initially 9 h per day but was progressively increased by an hour per week to reach 15.30 h in week 24.

Between 20 and 40 weeks of age, the birds were fed either the control (or basal) diet which contained the same vitamin E concentration as those found in commercial layer diets (10 mg DL-α-tocopherol acetate per kg) or an identical diet but supplemented with high doses of vitamin E : 125 or 500 mg of DL-α-tocopherol acetate. These layer diets were pelleted (pellets of 2.5 mm of diameter) and offered *ad libitum* throughout the experiment. The composition and characteristics of the basal diet are described in the Annexe (Table A2). These diets were allocated according to a blocked design in each room. In room 1, the temperature was maintained at 22°C for four weeks, then increased to 32°C for four weeks between weeks 24 and 28 and returned to 22°C for twelve weeks. In room 2, the temperature was maintained at 22°C for 12 weeks. It was then increased to 32°C for four weeks between weeks 32 and 36 and returned to 22°C for four weeks. The relative humidity was kept constant at 75%.

4.1.1.2. Measurements

During this period of 20 weeks, egg number, egg weight, food intake and body weight were measured as described in Chapter 3. Egg and eggshell weight, height, width and thickness were measured on all the eggs produced on three days in weeks 27 and 28 (end of the heat stress period in room 1), three days in weeks 31 and 32 (post-heat stress in room 1, pre-heat stress in room 2) and three days in weeks 35 and 36 (end of the heat stress period in room 2). Egg and eggshell weight were determined to the nearest 0.1 g and eggshell weight was determined on drained shells (with the inner and outer shell membranes) which had been dried for 48 hours in an oven at 110°C. Eggshell height and width were measured with a digital ruler (Wilmart) and

eggshell thickness was measured with another ruler (Mitsutoyo) on three points in the transverse aspect of the egg.

These parameters : egg weight (W), eggshell weight (w), length of the shell (L), width of the shell (l) allowed the calculation of the index of solidity of the shell (I). This index is represented by the ratio between the eggshell weight and the surface of the eggshell multiplied by 100. As the surface of the eggshell is a function of the weight of the egg, the index of solidity was calculated as follows (Sauveur, 1988b):

- if $W \leq 60$ g, $I = 100 * [w / (4.67 * W^{2/3})]$
- if $60 \text{ g} < W \leq 70$ g, $I = 100 * [w / (4.68 * W^{2/3}]$
- if $70 \text{ g} < W$, $I = 100 * [w / (4.69 * W^{2/3}]$

4.1.1.3. Statistical analyses

Individual egg production, feed intake, body weight were compared as described in Chapter 3. Eggshell parameters were compared on a four-week basis using a multifactorial analysis of variance (Anova). Separate statistical analyses was carried out for each 4-week period.

4.1.2. Results

In both rooms, egg production dropped significantly during the period of heat exposure (-18% in room 1 and -29% in room 2) compared with the previous 4-week period. As a few birds, equally distributed between the treatments (4, 3 and 3 for the groups given the diets containing 10, 125 and 500 mg vitamin E per kg respectively), died during and after heat stress, the number of residual degrees of freedom decreased from 196 at the beginning to 186 at the end of the experiment. Furthermore, as some birds also stopped laying (2, 3 and 0 for the same respective diets), the number of residual degrees of freedom was reduced in a greater extent for mean egg weight and food conversion efficiency and dropped from 196 to 181.

4.1.2.1. Room 1

4.1.2.1.1. Egg production and food intake

As was observed by Utomo *et al.* (1994), vitamin E supplementation did not affect egg production of birds which had not been exposed to a heat stress (first four weeks) (Table 10).

Table 10 : Egg production of laying hens fed vitamin E supplemented diets (10, 125 or 500 mg α-tocopherol/kg of diet, 50 birds per group) and subjected to a period of chronic heat stress between 24 and 28 weeks of age (room 1).

	Periods of experiment (weeks of age)*				
	20 - 24	***24 - 28***	*28 - 32*	*32 - 36*	*36 - 40*
Temperature (°C)	22	**32**	22	22	22
Egg production (%)					
10 mg/kg vit E	74.2	**56.2 a**	55.2 a	55.0 a	66.6 ab
125 mg/kg vit E	74.2	**61.1 a**	55.4 a	56.3 a	62.8 b
500 mg/kg vit E	76.2	**65.4 b**	64.4 b	66.7 b	72.6 a
Significance	NS	**P<0.05**	P<0.01	P<0.01	P<0.01
S.E.D.**	3.8	**4.2**	3.8	4.0	3.5
Mean egg weight (g)					
10 mg/kg vit E	52.2 a	**54.4**	56.6	59.4	62.4 a
125 mg/kg vit E	50.7 b	**53.0**	54.7	58.3	60.6 b
500 mg/kg vit E	51.1 ab	**53.6**	55.7	58.7	60.6 b
Significance	P<0.05	**NS**	NS	NS	P<0.05
S.E.D.**	0.6	**0.7**	0.8	0.9	0.8
Food intake (g/d)					
10 mg/kg vit E	79.5	**60.3**	77.6	85.9	94.8
125 mg/kg vit E	78.2	**60.0**	74.6	79.8	87.9
500 mg/kg vit E	80.0	**62.4**	80.8	85.4	94.2
Significance	NS	**NS**	NS	NS	NS
S.E.D.**	3.3	**3.6**	3.5	3.7	3.7
FCE * (g/g)**					
10 mg/kg vit E	0.43	**0.44 ab**	0.34	0.23 a	0.45
125 mg/kg vit E	0.46	**0.43 a**	0.30	0.32 ab	0.33
500 mg/kg vit E	0.47	**0.53 b**	0.43	0.43 b	0.46
Significance	NS	**P<0.05**	NS	P<0.05	NS
S.E.D.**	0.08	**0.04**	0.06	0.05	0.14

* Within a column, means of a given parameter indicated by different letters are statistically different. NS : Non significant (P>0.05).

** S.E.D. : Standard error of difference of means. Means were compared using Student's t test.

*** FCE : Food conversion efficiency (g of total egg mass per g of food).

During heat stress, the decline in egg production was less for the birds whose diets were supplemented with vitamin E, especially in the group receiving the 500 mg α-tocopherol per kg diet whose rate of egg production was 9.2% higher ($p<0.05$) during this period than in the control group (10 mg α-tocopherol per kg). During the post-stress period, the improvement in laying rate was maintained for two months with an average increase of 10.5% ($p<0.01$) compared to the control group. The group fed the 125 mg α-tocopherol per kg showed intermediate changes in egg production that did not differ significantly from controls.

The average egg weight and food intake declined during the heat stress exposure but no significant difference was apparent between the vitamin E supplemented and control groups. As a result, the total egg mass was increased for the birds receiving the supplemented diets. It varied from 30.7 g per hen per day for the control group to 35.1 g per hen per day for the group fed the 500 mg α-tocopherol per kg diet during the hot period; however, this difference failed to reach significance ($P<0.07$). During the two months post stress, the total egg masses for the same respective groups were 32.5 and 37.6 g per hen per day ($p<0.05$). Food conversion efficiency (total egg mass : food consumption, FCE) was improved, though the difference was not significant ($P<0.06$), during the stress period in the group fed the 500 mg α-tocopherol per kg diet compared to the control group. This difference in FCE was maintained during the two month-post-stress period.

4.1.2.1.2. Body weight

As the birds reached the peak of lay during the first four weeks of the experiment, their body weight was depressed (1,515 g versus 1,613 g in week 20 for the control group) (Table 11). In the group fed the 500 mg of vitamin E per kg diet, body weight was also depressed but to a lower extent (1,573 g versus 1,613 g in week 20). As a result, in week 24, the body weight of the birds fed the 500 mg of vitamin E diet was significantly higher than this of the birds fed the control diet (+3.8%, $P<0.05$). During heat stress, body weight markedly dropped in all the groups and the magnitude of the decrease was similar in all the groups (approximately 180 g). After heat stress, body weight tended to recover and was still similar in all the groups. These results demonstrated that vitamin E did not affect body weight during and after heat stress and that the increase in egg production observed in the supplemented groups was not obtained at the expense of the body weight of the birds.

Table 11 : Body weight of laying hens fed vitamin E supplemented diets (10, 125 or 500 mg a-tocopherol/kg of diet, 50 birds per group) and subjected to a period of chronic heat stress between 24 and 28 weeks of age (room 1).

	Periods of experiment (weeks of age)*					
	Week 20	*Week 24*	***Week 28***	*Week 32*	*Week 36*	*Week 40*
Temperature (°C)	22	22	**32**	22	22	22
Body weight (g)						
10 mg/kg vit E	1,613	1,515 a	**1,340**	1,392	1,432	1,484
125 mg/kg vit E	1,613	1,541 ab	**1,377**	1,437	1,433	1,522
500 mg/kg vit E	1,613	1,573 b	**1,382**	1,447	1,449	1,527
Significance	NS	P<0.05	**NS**	NS	NS	NS
S.E.D.**	3.8	3.8	**4.2**	3.8	4.0	3.5

* Within a column, means of a given parameter indicated by different letters are statistically different. NS : Non significant (P>0.05).

** S.E.D. : Standard error of difference of means. Means were compared using the Student's t test.

4.1.2.1.3. Eggshell characteristics

Eggshell thickness, which explains 75% of the eggshell solidity (Hunt and Voisey, 1966; Carter, 1969) was significantly reduced during the hot period (-6.3%, P<0.05) (Table 12) compared to this observed at the same time in room 2 in the control group (Table 15). Vitamin E supplementation did not affect this parameter during or after heat stress.

The index of solidity was also depressed during heat stress (-11.3%) compared to this on the control group in room 2 but was not affected by the vitamin E supplementation. As both of these parameters were not affected by the vitamin E supplementation, the increase in egg production observed during and after heat stress did not alter the thickness and solidity of the shell.

Table 12 : Eggshell characteristics of laying hens fed vitamin E supplemented diets (10, 125 or 500 mg α-tocopherol/kg of diet, 50 birds per group) and subjected to a period of chronic heat stress between 24 and 28 weeks of age (room 1).

	Periods of experiment (weeks of age)*		
	Week 27-28	*Week 31-32*	*Week 35-36*
Temperature (°C)	**32**	22	22
Eggshell thickness (μm)			
10 mg/kg vit E	**413**	443	438
125 mg/kg vit E	**412**	438	434
500 mg/kg vit E	**412**	430	425
Significance	**NS**	NS	NS
S.E.D.**	**4.2**	3.8	4.0
Index of solidity (g x cm^{-2} x 100)			
10 mg/kg vit E	**7.55**	8.47	8.56
125 mg/kg vit E	**7.52**	8.30	8.59
500 mg/kg vit E	**7.62**	8.23	8.37
Significance	**NS**	NS	NS
S.E.D.**	**4.2**	3.8	4.0

* Within a column, means of a given parameter indicated by different letters are statistically different. NS : Non significant (P>0.05).

** S.E.D. : Standard error of difference of means. Means were compared using Student's t test.

4.1.2.2. Room 2

4.1.2.2.1. Egg production and food intake

Similarly as in room 1, vitamin E did not affect any of the egg production or food intake characteristics before heat stress (Table 13). During and after heat stress, the groups fed the vitamin E supplemented diets showed greater egg production than the control group. However, the differences failed to reach significance ($P<0.10$ during the stress period and $P<0.07$ after the stress). Mean egg weight and food intake were not affected by the vitamin E treatment during and after heat stress. FCE was improved in the vitamin E supplemented groups during and after heat stress, although the difference was not significant. Therefore, although the magnitude of the differences with the control groups were not as great as in room 1, the vitamin E treatment improved egg production and FCE in room 2 where the birds were stressed later in lay.

Table 13 : Egg production of laying hens fed vitamin E supplemented diets (10, 125 or 500 mg α-tocopherol/kg of diet, 50 birds per group) and subjected to a period of chronic heat stress between 32 and 36 weeks of age (room 2).

	Periods of experiment (weeks of age)*				
	20 - 24	*24 - 28*	*28 - 32*	*32 - 36*	*36 - 40*
Temperature (°C)	22	22	22	**32**	22
Egg production (%)					
10 mg/kg vit E	77.6	82.0	74.0	**44.7**	47.8
125 mg/kg vit E	75.4	83.0	78.6	**48.1**	52.1
500 mg/kg vit E	78.7	80.5	74.5	**49.9**	53.9
Significance	NS	NS	NS	**NS**	NS
S.E.D.**	3.8	4.2	3.8	**4.0**	3.5
Mean egg weight (g)					
10 mg/kg vit E	52.7 a	56.2	58.1	**57.4**	59.7
125 mg/kg vit E	51.0 b	55.3	57.6	**56.9**	59.4
500 mg/kg vit E	51.0 b	55.2	57.8	**57.4**	59.3
Significance	P<0.05	NS	NS	**NS**	NS
S.E.D.**	0.6	0.7	0.8	**0.9**	0.8
Food intake (g/d)					
10 mg/kg vit E	85.5	89.8	84.6	**55.5**	77.7
125 mg/kg vit E	82.7	92.7	89.4	**57.0**	80.4
500 mg/kg vit E	82.7	89.5	86.0	**60.6**	80.8
Significance	NS	NS	NS	**NS**	NS
S.E.D.**	3.3	3.6	3.5	**3.7**	3.7
FCE * (g/g)**					
10 mg/kg vit E	0.45	0.49	0.50	**0.39**	0.25
125 mg/kg vit E	0.34	0.47	0.50	**0.44**	0.34
500 mg/kg vit E	0.46	0.47	0.50	**0.42**	0.37
Significance	NS	NS	NS	**NS**	NS
S.E.D.**	0.08	0.04	0.06	**0.05**	0.14

* NS : Non significant (P>0.05).

** S.E.D. : Standard error of difference of means. Means were compared using Student's t test.

*** FCE : Food conversion efficiency (g of total egg mass per g of food).

4.1.2.2.2. Body weight

Between weeks 24 and 28, the birds fed the control diet showed a greater reduction in their body weight than the birds fed the 125 and 500 mg of vitamin E per kg diets showed an increased body weight compared to the birds fed the control diet (Table 14). During the stress period,

body weight declined in all groups but the magnitude of it was reduced in the birds fed the diet containing 500 mg of vitamin E per kg diet. As a result, body weight was higher during heat stress and recovered faster after heat stress in both supplemented groups. Again the greater egg production allowed by the vitamin E supplementation was not obtained in this room was not obtained at the expense of the body weight of the birds.

Table 14 : Body weight of laying hens fed vitamin E supplemented diets (10, 125 or 500 mg α-tocopherol/kg of diet, 50 birds per group) and subjected to a period of chronic heat stress between 32 and 36 weeks of age (room 2).

	Weeks of experiment (weeks of age)*					
	Week 20	*Week 24*	*Week 28*	*Week 32*	***Week 36***	*Week 40*
Temperature (°C)	22	22	22	22	**32**	22
Body weight (g)						
10 mg/kg vit E	1,611	1,584	1,536 a	1,494 a	**1301 a**	1,427 a
125 mg/kg vit E	1,611	1,619	1,605 b	1,586 b	**1,385 b**	1,524 b
500 mg/kg vit E	1,612	1,573	1,557 ab	1,522 ab	**1,381 b**	1,484 ab
Significance	NS	NS	P<0.05	P<0.05	**P<0.05**	P<0.05
S.E.D.**	3.8	3.8	4.2	3.8	**4.0**	3.5

* Within a column, means of a given parameter affected by different letters are statistically different. NS : Non significant (P>0.05).

** S.E.D. : Standard error of difference of means. Means were compared using Student's t test.

4.1.2.2.3. Eggshell characteristics

Eggshell thickness was significantly reduced during the hot period (-10.5%, P<0.05) (Table 15) compared to the thickness observed pre-stress or at the same age in room 1 (Table 12). Vitamin E supplementation did not affected eggshell thickness before and during the stress period. The index of solidity was depressed during heat stress (-6.7%) but was not affected by the vitamin E supplementation.

Table 15 : Eggshell characteristics of laying hens fed vitamin E supplemented diets (10, 125 or 500 mg α-tocopherol/kg of diet, 50 birds per group) and subjected to a period of chronic heat stress between 32 and 36 weeks of age (room 2).

	Periods of experiment (weeks of age)*		
	Week 27-28	*Week 31-32*	***Week 35-36***
Temperature (°C)	22	22	**32**
Eggshell thickness (μm)			
10 mg/kg vit E	441	428	**392**
125 mg/kg vit E	445	423	**365**
500 mg/kg vit E	444	421	**371**
Significance	NS	NS	**NS**
S.E.D.**	4.2	3.8	**4.0**
Index of solidity (g x cm^{-2} x 100)			
10 mg/kg vit E	8.16	8.48	**7.47**
125 mg/kg vit E	8.05	8.27	**7.35**
500 mg/kg vit E	8.01	8.34	**7.45**
Significance	NS	NS	**NS**
S.E.D.**	4.2	3.8	**4.0**

* Within a column, means of a given parameter indicated by different letters are statistically different. NS : Non significant (P>0.05).

** S.E.D. : Standard error of difference of means. Means were compared using Student's t test.

4.1.3. Discussion

The results of experiment 1 showed that supplementing diets with a relatively high concentration of α-tocopherol (500 mg per kg) reduced the detrimental effect of chronic heat stress on egg production in laying hens. The average benefit during the 4-week stress period was 7% (9% and 5% in rooms 1 and 2 respectively). Although the effect of vitamin E was not as great as in Utomo *et al.* (1994) experiment, these results probably give a more reliable indication of the benefit of vitamin E as experiment 1 was carried on a larger scale. Furthermore, and similarly as was observed by Utomo *et al.* (1994), the overall benefits to egg production in both rooms extended beyond these periods of stress, because treated hens returned to normal levels of production sooner after the removal of the heat stress.

It should however be pointed out at this stage that, in both rooms, the daily food consumption was relatively low. Indeed, when the birds were aged between 20 and 24 weeks, the food intake

averaged 79.5 and 85.5 g/day in rooms 1 and 2 for the controls groups. These values are below the ISA recommendations (ISA Guide, 1993b) for birds of a similar age (110 g/day). This reduced food intake could raised some questions about the validity of the results obtained in this experiment. However, numerous authors have demonstrated that birds receiving limited amounts of feed during rearing or during a heat wave gave better production than birds fed *ad libitum* (Connor, 1980; Gogny and Souilem, 1991). Lighter birds were also found to have less of a problem with heat stress since they have more surface area for heat dissipation per unit weight (Teeter, 1991). The reduced food intake observed in this experiment should therefore have led to an improved resistance to heat stress in all groups of birds, nevertheless non-supplemented birds showed a much lower egg production than vitamin E-supplemented ones. This experiment therefore suggested that vitamin E had a positive effect in heat-stressed birds and, in order to confirm this result, another similar experiment was carried out (Experiment 2).

In contrast with the findings of Utomo *et al.* (1994), vitamin E did not affect egg weight during the period of stress. The difference in response is probably not related to age of hen, since hens in Utomo *et al.* (1994) experiment were stressed at about the same age as those in room 2 in experiment 1. Instead, the difference may be related to the duration of the stress or to egg weight. Indeed, the latter was considerably higher before the stress in Utomo *et al.* (1994) experiment (66 g) than in experiment 1 (52 and 58 g in rooms 1 and 2 respectively), even though the hens were from the same strain.

Total egg mass over the stress period was significantly higher with the highest dietary vitamin E concentration. Food intake was also higher than in the control group with this treatment, though the difference was relatively smaller and not statistically significant. The overall effect was an improvement in FCE with the vitamin E treatment, which represents an additional commercial advantage to the use of vitamin E under conditions of heat stress. Furthermore, the greater number of eggs produced by the vitamin E supplemented birds during heat stress did not induce the production of more fragile eggs as both the thickness and index of solidity of the shell were similar in all the groups.

A dose of 500 mg of α-tocopherol per kg of diet was therefore seemed to be efficient in alleviating the depressive effect of heat stress on a large number of laying hens. However this dose of supplementation is quite high as it represents approximately 50 times the amount usually incorporated in standard commercial layer diets.

4.2. Experiment 2

The objective of experiment 2 was to determine the most cost-effective dose and duration for feeding of vitamin E. Different dietary vitamin E concentrations were tested on laying hens exposed to the same type of stress as in experiment 1 (four weeks at 32°C) and at the age of 26 weeks. Five doses of vitamin E were tested : 10 (control), 125, 250, 375 and 500 mg α-tocopherol per kg diet. To define the most effective duration of supplementation, two modes of distribution were also compared : either the different doses of vitamin E were only provided for a short duration (four weeks before the stress period) and then the birds were fed the control diet or provided for a long duration (four weeks before, during and after heat stress).

4.2.1. Materials and methods

4.2.1.1. Selection of the birds and husbandry

Five hundred ISA Brown hens aged 16 weeks were housed in individual cages in two climate rooms at 22°C for an adaptation period of six weeks. Between 16 and 22 weeks, the pullets were fed pre-lay diets for which composition and characteristics are described in the Annexe (Table A3). Between 20 and 22 weeks of age, egg production was recorded daily and at 22 weeks of age, the birds were weighed individually. Uniformity of egg production between 20 and 22 weeks of age and live-weight at 22 weeks of age were used as criteria to select 335 birds. These birds were then housed in individual cages in three-tier battery units in two temperature controlled rooms. The light period was initially 9 h per day but was progressively increased by an hour per week from week 18 to reach 15.30 h in week 24. In both rooms the climatic conditions were identical: the temperature was maintained at 22°C for four weeks, then increased to 32°C for four weeks between 26 and 30 weeks of age (stress period) and returned to 22°C for eight weeks. The relative humidity, actively controlled, was kept constant at 75%.

The basal laying diet was a pelleted diet (pellets of 2.5 mm of diameter), its composition and characteristics are given in the Annexe (Table A4). The control diet was the basal diet containing 10 mg DL-α-tocopherol acetate per kg and other experimental diets were obtained by supplementing the basal diet with 125, 250, 375 or 500 mg of DL-α-tocopherol acetate (hyper-supplemented diets). From 22 to 26 weeks, each diet was fed to groups of 67 birds arranged in a random blocked design in each room. At the end of week 26, 5 birds were killed in each group. From week 26 to the end of the experiment, half of the birds in each group were fed on the

control diet (short supplementation duration or SSD groups). The remaining birds in each group continued to receive the same diets as before the stress until the end of the experiment (long supplementation duration or LSD groups). At the end of week 30, 10 birds were killed in the control group and 5 birds were killed in each SSD or LSD group. The total number of remaining birds was therefore 260 (52 birds in the control group and 26 birds in each SSD or LSD groups).

4.2.1.2. Measurements

Egg production, food intake and body weight were determined on the 260 birds kept throughout the experiment (52 birds in the control group and 26 birds per treatment in the other groups) and measured as described in Chapter 3. In addition, yolk weight was measured 3 times per week on the eggs laid by the same 10 birds in the control group and 5 birds in each of the other groups. Blood samples were collected from 10 birds in the control group and 5 birds/treatment in the other groups prior to heat stress (end of the 26th week of age), twice during heat stress (end of the 27th and 30th weeks of age) and twice after heat stress (end of the 31st and 34th weeks of age). The 25 and 50 birds killed either prior to heat stress (end of the 26th week of age) or during heat stress (30th week of age) were dissected and their livers were removed. Plasma and liver samples were stored at -80°C before analysis for α-tocopherol.

4.2.1.3. Analyses

Boiling has been shown to have no effect on yolk weight (Bougon *et al.*, 1982), hence yolk weight was measured on hard yolks after boiling the eggs for 12 minutes. Plasma vitamin E concentration was measured by high pressure liquid chromatography after a double extraction with 10% ethylacetate in hexane without saponification. Liver vitamin E concentration was determined after saponification of liver homogenate with an ethanolic potassium hydroxide solution maintained at reflux for 30 minutes (in presence of ascorbic acid). Once the saponification medium had cooled, it was diluted with distilled water and vitamin E was extracted with hexane. After evaporation of hexane, vitamin E concentration was determined by high pressure liquid chromatography with U.V. detection.

Statistical analyses were carried out on individual egg production, food intake, body weight as described in Chapter 3. A separate anova was carried out for each 4-week period to compare yolk weight : egg weight ratios. For each plasma or liver vitamin E concentration, abnormal data

were determined using Student t-test and discarded. Means were compared using by analysis of variance and Student t-test from Genstat 5 (Lawes Agricultural Trust).

4.2.2. Results

4.2.2.1. Egg production

During the first four weeks of the experiment (22°C), egg production was very similar in all groups and not affected by the vitamin E treatment (Table 16).

Table 16 : Egg production (number of eggs per hen per day x 100) of laying hens fed vitamin E supplemented diets (10, 125, 250, 375 or 500 mg α-tocopherol/kg of diet, 26 birds per group) and subjected to a period of chronic heat stress between 26 and 30 weeks of age.

		Periods of experiment (weeks of age)			
		22 - 26	***26 - 30***	*30 - 34*	*34 - 38*
Temperature (°C)		22	**32**	22	22
Control - 10 mg/kg		90.1	**68.9**	62.7	80.4
S.S.D.	125 - 10 mg/kg	90.4	**71.2**	68.7	78.3
	250 - 10 mg/kg	89.8	**73.9**	70.7	86.7
	375 -10 mg/kg	90.6	**72.8**	71.0	87.5
	500 -10 mg/kg	90.7	**75.6**	70.8	83.6
	Mean	90.4	**73.4**	70.3 *	84.0
L.S.D.	125 mg/kg	90.4	**76.5**	66.2	85.8
	250 mg/kg	89.8	**80.6** **	75.3 **	86.1
	375 mg/kg	90.6	**74.6**	75.0 **	88.3 *
	500 mg/kg	90.7	**77.1**	75.7 ***	86.3
	Mean	90.4	**77.2** **	73.0 ***	86.6 **
S.E.D. (1)		2.4	**4.7**	5.0	3.5
S.E.D. (2)		-	**3.3**	3.5	2.5

*, **, *** : Within a column, values indicated by (*,**,***) differ significantly from the control group (P<0.05, P<0.02, P<0.01).

S.S.D. : Short Supplementation Duration (between 22 and 26 weeks of age)

L.S.D. : Long Supplementation Duration (between 22 and 38 weeks of age)

S.E.D. (1) : Standard error of difference between the control and each of the S.S.D. or L.S.D. groups.

S.E.D. (2) : Standard error of difference between the control and the mean of the S.S.D. or L.S.D. groups.

During the four following weeks (32°C), egg production dropped significantly in the group fed the control diet (-21.2%, P<0.01). The magnitude of this decrease was lower in the SSD groups and egg production was higher by 4.5% (P<0.18) compared to the control group. In the SSD groups, the best egg production was obtained in the group fed 500 mg vitamin E per kg (+6.7%, P<0.16). Egg production also fell in the LSD groups during the heat stress period, but the magnitude of the decrease was less than in the SSD groups and egg production was higher by +8.3% (P<0.02) in the LSD groups compared to the control group. The best egg production was obtained in the LSD group fed 250 mg vitamin E per kg (+11.7%, P<0.02). During the four weeks following heat stress, egg production was also better in the birds receiving the hyper-supplemented diets (+7.6%, P<0.04, in the SSD groups and +10.3%, P<0.01, in the LSD groups) compared to the control group. Among the SSD groups, best egg production was seen in the group fed pre-stress on the diet containing 375 mg vitamin E per kg (+8.3%, P<0.10). In the LSD groups, significant improvements were obtained in the groups fed 250, 375 and 500 mg vitamin E per kg (+12.6%, P<0.02; +12.3%, P<0.02 and +13.0%, P<0.01 respectively).

4.2.2.2. Mean egg weight

Table 17 : Mean egg weight (g per egg) of laying hens fed vitamin E supplemented diets (10, 125, 250, 375 or 500 mg α-tocopherol/kg of diet, 26 birds per group) and subjected to a period of chronic heat stress between 26 and 30 weeks of age.

		Periods of experiment (weeks of age)			
		22 - 26	***26 - 30***	*30 - 34*	*34 - 38*
Temperature (°C)		22	**32**	22	22
Control - 10 mg/kg		55.8	**56.7**	58.7	63.3
S.S.D.	125 - 10 mg/kg	55.5	**56.8**	59.3	63.3
	250 - 10 mg/kg	54.7	**55.4**	58.0	62.6
	375 -10 mg/kg	55.8	**57.3**	60.0	64.3
	500 -10 mg/kg	54.9	**55.6**	57.2	61.1 *
	Mean	55.2	**56.3**	58.6	62.8
L.S.D.	125 mg/kg	55.5	**56.0**	57.8	62.0
	250 mg/kg	54.7	**56.3**	58.7	62.0
	375 mg/kg	55.8	**56.3**	59.3	63.5
	500 mg/kg	54.9	**55.8**	57.7	61.2 *
	Mean	55.2	**56.1**	58.3	62.2
S.E.D. (1)		0.7	**0.9**	1.0	1.0
S.E.D. (2)		-	**0.7**	0.7	0.7

* : For notes, see Table 16.

As observed in experiment 1, mean egg weight was not affected by the vitamin E treatment before during or after heat stress (Table 17).

4.2.2.3. Food intake

Food intake was depressed by heat stress but was higher during the stress and immediate post-stress periods in birds fed the hyper supplemented diets (Table 18). The improvement was particularly noticeable in the LSD groups post-stress (+8.5%, P<0.02, relative to the control group).

Table 18 : Food intake (g per hen per day) of laying hens fed vitamin E supplemented diets (10, 125, 250, 375 or 500 mg α-tocopherol/kg of diet, 26 birds per group) and subjected to a period of chronic heat stress between 26 and 30 weeks of age.

		Periods of experiment (weeks of age)			
		22 - 26	***26 - 30***	*30 - 34*	*34 - 38*
Temperature (°C)		22	**32**	22	22
Control - 10 mg/kg		98.1	**69.4**	85.8	104.9
S.S.D.	125 - 10 mg/kg	98.9	**73.0**	89.3	104.4
	250 - 10 mg/kg	99.4	**73.5**	90.1	108.6
	375 -10 mg/kg	100.0	**74.0**	93.2	109.0
	500 -10 mg/kg	103.7	**72.3**	88.0	103.8
	Mean	100.5	**73.2**	90.2	106.5
L.S.D.	125 mg/kg	98.9	**72.3**	88.0	105.3
	250 mg/kg	99.4	**76.2**	93.6	105.5
	375 mg/kg	100.0	**71.5**	93.2	107.5
	500 mg/kg	103.7	**75.7**	97.6 ***	107.3
	Mean	100.5	**73.9**	93.1 **	106.4
S.E.D. (1)		3.5	**3.8**	4.4	3.9
S.E.D. (2)		-	**2.7**	3.1	2.8

, * : For notes, see Table 16.

4.2.2.4. Food conversion efficiency

FCE was improved during heat stress (+20.8%, P<0.05) and in the four weeks following heat stress and (+21.1%, P<0.05) in the LSD 250 mg vitamin E per kg group compared to the control

group (Table 19). In the SSD groups and other LSD groups, FCE was also improved during and after heat stress compared to the control group but not significantly.

Table 19 : Food conversion efficiency (g of egg per g of food) of laying hens fed vitamin E supplemented diets (10, 125, 250, 375 or 500 mg α-tocopherol/kg of diet, 26 birds per group) and subjected to a period of chronic heat stress between 26 and 30 weeks of age.

		Periods of experiment (weeks of age)			
		22 - 26	***26 - 30***	*30 - 34*	*34 - 38*
Temperature (°C)		22	**32**	22	22
Control - 10 mg/kg		0.51	**0.48**	0.38	0.46
S.S.D.	125 - 10 mg/kg	0.51	**0.50**	0.42	0.44
	250 - 10 mg/kg	0.49	**0.51**	0.41	0.49
	375 -10 mg/kg	0.51	**0.52**	0.39	0.51 **
	500 -10 mg/kg	0.47	**0.53**	0.43	0.48
	Mean	0.49	**0.51**	0.41	0.48
L.S.D.	125 mg/kg	0.51	**0.56**	0.40	0.50 *
	250 mg/kg	0.49	**0.58** *	0.46 **	0.50
	375 mg/kg	0.51	**0.54**	0.44	0.51 ***
	500 mg/kg	0.47 ***	**0.51**	0.42	0.48
	Mean	0.49	**0.55** *	0.43 *	0.50 ***
S.E.D. (1)		0.01	**0.04**	0.03	0.02
S.E.D. (2)		-	**0.03**	0.02	0.01

*, **, *** : For notes, see Table 16.

4.2.2.5. Body weight

Body weight decreased during the period of heat stress and the decrease was greater in the control then in the SSD or LSD treatments (Table 20). In the LSD 500 mg vitamin E per kg group, body weight was higher during heat stress (+6.9%, $P<0.05$) and during the four weeks following heat stress (+7.1%, $P<0.05$) compared to the control group.

Table 20 : Body weight (g per hen) of laying hens fed vitamin E supplemented diets (10, 125, 250, 375 or 500 mg α-tocopherol/kg of diet, 26 birds per group) and subjected to a period of chronic heat stress between 26 and 30 weeks of age.

		Weeks of experiment (weeks of age)			
		26	***30***	*34*	*38*
Temperature (°C)		22	**32**	22	22
Control - 10 mg/kg		1613	**1440**	1539	1670
S.S.D.	125 - 10 mg/kg	1621	**1471**	1556	1662
	250 - 10 mg/kg	1638	**1479**	1591	1711
	375 -10 mg/kg	1628	**1476**	1594	1678
	500 -10 mg/kg	1669	**1471**	1547	1672
	Mean	1639	**1474**	1572	1681
L.S.D.	125 mg/kg	1621	**1451**	1556	1654
	250 mg/kg	1638	**1469**	1584	1676
	375 mg/kg	1628	**1465**	1579	1686
	500 mg/kg	1669	**1534** *	1647 *	1722
	Mean	1639	**1480**	1591	1685
S.E.D. (1)		35	**43**	48	59
S.E.D. (2)		-	**30**	34	42

* : For notes, see Table 16.

4.2.2.6. Ratio egg yolk weight : egg weight

The ratio yolk weight : egg weight was not depressed by heat stress and rose from 21.5% at the beginning of the experiment to 23.5% at the end in the control group (Table 21). There was no effect of the level of vitamin E supplementation before, during or after heat stress.

Table 21 : Ratio egg yolk weight per egg weight (g per g) of laying hens fed vitamin E supplemented diets (10, 125, 250, 375 or 500 mg α-tocopherol/kg of diet, 26 birds per group) and subjected to a period of chronic heat stress between 26 and 30 weeks of age.

		Periods of experiment (weeks of age)			
		22 - 26	***26 - 30***	*30 - 34*	*34 - 38*
Temperature (°C)		22	**32**	22	22
Control - 10 mg/kg		0.215	**0.226**	0.223	0.235
S.S.D.	125 - 10 mg/kg	0.218	**0.226**	0.224	0.242
	250 - 10 mg/kg	0.210	**0.227**	0.221	0.229
	375 -10 mg/kg	0.212	**0.226**	0.225	0.234
	500 -10 mg/kg	0.218	**0.231**	0.224	0.240
	Mean	0.215	**0.227**	0.223	0.236
L.S.D.	125 mg/kg	0.218	**0.233**	0.223	0.239
	250 mg/kg	0.210	**0.226**	0.217	0.233
	375 mg/kg	0.212	**0.223**	0.221	0.233
	500 mg/kg	0.218	**0.227**	0.225	0.233
	Mean	0.215	**0.227**	0.221	0.235
S.E.D. (1)		0.005	**0.007**	0.006	0.008
S.E.D. (2)		-	**0.005**	0.004	0.006

For notes, see Table 16.

4.2.2.7. Concentration of vitamin E in the plasma

The concentration of vitamin E in the plasma was proportional to the dietary concentration during the pre-stress period (Table 22). During heat stress, in the SSD groups, as the birds were fed the control diet, the plasma concentration of vitamin E dropped markedly and showed little residual effect of earlier dietary treatment. From 30 weeks, in all the SSD groups, the vitamin E concentrations were uniformly low. Plasma vitamin E concentrations remained much higher in the LSD groups during stress and post-stress periods. Although they were reduced by 39% in the LSD groups during the stress period (weeks 27 and 30 combined) compared to before the stress, they recovered in the post heat-stress period and were only slightly lower than before the stress (-7%, weeks 31 and 34 combined). The determination of the intake of vitamin E in the birds whose plasma was analysed for vitamin E concentration revealed that, in the LSD groups, vitamin E intake was reduced by 29% during the stress period and by 11% during the post-heat stress period compared to before the stress.

Table 22 : Plasma concentration of vitamin E (μg per ml) of laying hens fed vitamin E supplemented diets (10, 125, 250, 375 or 500 mg α-tocopherol/kg of diet, 10 birds per group before heat stress, 5 birds per group thereafter) and subjected to a period of chronic heat stress between 26 and 30 weeks of age.

		Periods of experiment (weeks of age)				
		Week 26	***Week 27***	***Week 30***	*Week 31*	*Week 34*
Temperature (°C)		22	**32**	**32**	22	22
Control - 10 mg/kg		10	**13**	**10**	11	7
S.S.D.	125 - 10 mg/kg	46 ***	**12**	**8**	7	11
	250 - 10 mg/kg	94 ***	**10**	**9**	8	7
	375 -10 mg/kg	134 ***	**25**	**11**	16	9
	500 -10 mg/kg	172 ***	**33**	**7**	8	7
	Mean	111	**20**	**9**	10	8
L.S.D.	125 mg/kg	46 ***	**59** ***	**28**	68 **	52 ***
	250 mg/kg	84 ***	**43**	**55** **	75 ***	72 ***
	375 mg/kg	134 ***	**71** ***	**71** ***	102 ***	151 ***
	500 mg/kg	172 ***	**55** ***	**160** ***	115 ***	194 ***
	Mean	111	**57** ***	**79** ***	90 ***	117 ***
S.E.D. (1)		12	**15**	18	**22**	11
S.E.D. (2)		-	**10**	13	15	8

, * : For notes, see Table 16.

4.2.2.8. Concentration of vitamin E in the liver

Before heat stress, the concentration of vitamin E was elevated at higher dietary concentrations of vitamin E at week 26 (Table 23). During heat stress, the concentration of vitamin E fell markedly in the liver of SSD birds, and was similar as the value observed in the control group in all SSD groups by week 30. In the LSD groups, liver concentration was only slightly depressed (-18%) at week 30 compared to before the stress.

Table 23 : Liver concentration of vitamin E (μg per g) of laying hens fed vitamin E supplemented diets (10, 125, 250, 375 or 500 mg α-tocopherol/kg of diet, 5 birds per group) and subjected to a period of chronic heat stress between 26 and 30 weeks of age.

	Periods of experiment (weeks of age)		
	Week 26	*Week 30*	
Temperature (°C) - Groups	22	**32 SSD**	**32 LSD**
Control - 10 mg/kg	4	**5**	**5**
125 - 10 mg/kg	47	**3**	**42** ***
250 - 10 mg/kg	70 *	**4**	**92** ***
375 -10 mg/kg	84 ***	**1**	**67** ***
500 -10 mg/kg	249 ***	**4**	**167** ***
Mean	112	**3**	**92** ***
S.E.D. (1)	26	**8**	
S.E.D. (2)	-	**5**	

*, *** : For notes, see Table 16.

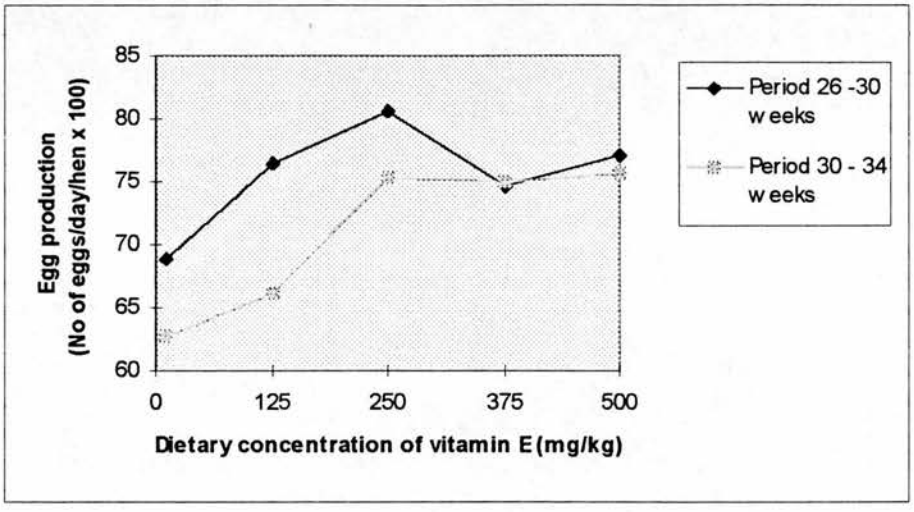
4.2.3. Discussion

This experiment supported the findings in experiment 1 which demonstrated that a high dietary supplementation with vitamin E alleviates the detrimental effects of heat stress on egg production. It also suggested that, for best results, the supplementation has to be provided not only before the stress but also during and after the stress. Thus, although egg production during and after heat stress was improved by feeding extra vitamin E only pre-stress (SSD groups), egg production was superior in those groups maintained on the high vitamin E diets throughout the experiment (LSD groups). The magnitude of the improvements of egg production during heat stress were also in accordance with the previous findings as increases of 8.2% and 9.2% were obtained with a diet containing 500 mg vitamin E per kg in the present and previous experiment (Experiment 1).

The objective of the present study was to determine the optimum dietary concentration of vitamin E for the improvement of egg production during heat stress. The overall response of egg production during the stress and post-stress periods in the LSD groups to the different dietary doses of vitamin E suggested that 125 mg per kg did not give maximum performance but that there was no obvious consistent benefit to supplementation above 250 mg vitamin E per kg. Thus mean egg productions over the period 26 to 38 weeks were 76.2, 80.7, 79.3 and 79.7% for

the groups fed 125, 250, 375 and 500 mg per kg respectively. It is therefore concluded that the alleviation of effects of chronic heat stress on egg production is optimised by dietary supplementation with 250 mg vitamin E per kg (Figure 17).

Figure 17 : Relationship between egg production (No of eggs per hen per day x 100) and dietary dose of vitamin E (mg per kg) of laying hens fed vitamin E supplemented diets (10, 125, 250, 375 or 500 mg α-tocopherol/kg of diet, 26 birds per group) during and after heat stress.

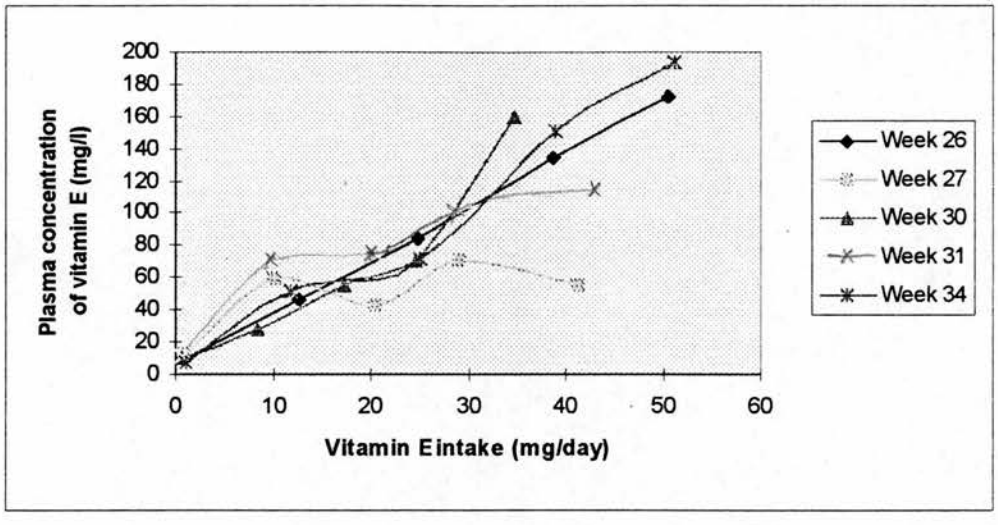


Although still slightly low, the average food intake before the period of stress was much higher in the present experiment (average of 100 g/day) compared to that observed in Experiment 1 (80 g/day). Despite this difference, similar increases in egg production were observed in both experiments during the stress period in birds which received similar levels of vitamin E supplementation. It therefore appears that the relatively low food intake observed in Experiment 1 did not result in inappropriate increases in egg production in vitamin E-supplemented birds. As was also observed in Experiment 1, vitamin E supplementation had no effect on egg weight or food intake. Both total egg mass and FCE were therefore significantly improved during and after heat stress in the LSD groups fed 250 mg vitamin E per kg or more and these improvements were not obtained at the expense of the body weight of the birds. The absence of any depressive effect of vitamin E on the ratio of yolk weight to egg weight during heat stress was also consistent with our previous findings. As vitamin E treatment was shown not to affect egg weight, it suggested that yolk weight was maintained and that total egg yolk mass was increased during and after heat stress in the birds fed the diets containing high doses of vitamin E.

Under thermoneutral conditions during the pre-stress period (week 26), plasma vitamin E concentration showed a highly linear relationship with dietary vitamin E concentration. This is

consistent with previous findings (Pudelkiewicz and Mary, 1969) who showed a linear relationship up to a dietary concentration of 667 mg *dl*-α tocopherol per kg. During heat stress, this relationship was maintained in the LSD groups as plasma concentration of vitamin E decreased broadly in proportion to vitamin E intake (Figure 18). In the SSD groups, which were transferred to the control diet, plasma concentrations of vitamin E rapidly fell and were similar to the level observed in the control group at the end of the stress period.

Figure 18 : Relationship between plasma concentration of vitamin E (µg per ml) and vitamin E intake (mg per day) of laying hens at different periods during the experiment : before (week 26), during (weeks 27 and 30) and after (weeks 31 and 34) heat stress.



Liver concentration of vitamin E was also elevated by higher dietary vitamin E concentrations. Liver vitamin E concentrations in the LSD groups declined upon imposition of the heat stress, but only in broad proportion to the depression in food intake. There was no evidence to suggest that heat stress had a specific effect in depressing vitamin E status in hens. In the SSD groups, plasma and liver concentrations of vitamin E declined markedly after the birds were transferred to the control diet, so that after 4 weeks there was no residual effect of the initial dietary content on vitamin E circulation or liver reserves. These findings on the rapid depletion of vitamin E explain why the heat stress had a more severe effect on the SSD groups and emphasise the importance of maintaining an adequate supply of dietary vitamin E throughout the period when birds are exposed to and recovering from heat stress.

This experiment therefore suggested that providing 250 mg vitamin E per kg throughout the experiment was adequate to alleviate significantly the depressive effect of heat stress on egg

production. Dietary supplementation with this dose of vitamin E was also proved to be sufficient to reduce the drop in the concentration of vitamin E in the plasma and in the liver during heat stress. This may provide an adequate pool of vitamin E in the circulation and in the liver to overcome the effect of heat stress. As was observed by McIntosh *et al.* (1993), supplementation with vitamin E may reduce the free radical attack, lipid peroxidation and membrane damage of cellular and organelle membranes. Vitamin E-mediated protection of hepatocyte membranes may explain the improved production or export of egg yolk precursors from the liver and therefore the increase in egg production during heat stress.

4.3. Conclusion

In conclusion, despite the relatively low food intake, Experiment 1 confirmed the observations of Utomo *et al.* (1994) and demonstrated that a dose of 500 mg vitamin E per kg diet alleviated the detrimental effect of a chronic heat stress (4 weeks at 32°C) on egg production in laying hens. Although not as great as in the experiment of Utomo *et al.* (1994), the increase in the number of eggs produced during and after heat stress in the 500 mg vitamin E per kg diet compared to the control group was probably more reliable as the experiment was carried on a greater number of birds. Furthermore, this experiment showed that the increase in egg production was significant both during heat stress (+9.2%, $P<0.05$) and in the eight weeks of recovery at 22°C (+10.5%, $P<0.01$). The other tested dose of 125 mg vitamin E per kg diet showed that this dose, although also leading to an increase in egg production, was not sufficient to alleviate the effect of heat stress in a significative way. Experiment 1 also demonstrated a greater response in egg production in birds stressed at the peak of lay rather than later in lay.

Experiment 2 supported the positive effect of vitamin E on egg production during and after heat stress and suggested that a dose of 250 mg vitamin E/kg diet, if distributed continuously before, during and after heat stress, is sufficient to alleviate significantly the adverse effects of heat stress on egg production. The increase in the number of eggs produced with this dose reached +11.7% ($P<0.02$) during heat stress and +12.6% ($P<0.02$) during the four weeks following heat stress.

In the next chapters, the mechanism(s) by which vitamin E alleviates the detrimental effects of heat stress on egg production were investigated on birds exposed to chronic heat stress around the peak of lay and receiving a dietary dose of approximately 250 to 300 mg vitamin E per kg diet.

Chapter 5

Development and validation of a technique to measure the uptake of vitellogenin by oocytes *in vivo*

During chronic heat stress, egg production and total yolk mass are reduced. Previous studies by Utomo *et al.* (1994) have indicated that the decrease in egg production is associated with reduced plasma concentration of the two main egg yolk precursors, triglyceride-rich very low density lipoprotein (VLDL) and vitellogenin. A reduction in egg production could be explained, at least in part, by the reduction in the plasma concentration of egg yolk precursors. There may also be a simultaneous effect of heat stress on the uptake of these precursors into the oocytes. This additional effect has therefore to be assessed to have a more complete understanding of the mechanisms by which heat stress and vitamin E affect egg production.

Taking advantage of the high affinity metal binding property of phosvitins and lipovitellins (section 2.1.1.1.), vitellogenin was labelled with a radioactive metal and chosen as an indicator of the uptake of yolk precursors by oocytes. As vitellogenin accounts for about 90% of the protein-bound zinc, ^{65}Zn, an easily detectable γ-emitter, was used to label vitellogenin. Once the radiolabelled protein was prepared, it was injected to birds and, after a given period of time, the radioactivity found in the oocytes was measured to determine their uptake in vitellogenin-bound ^{65}Zn. This chapter describes how a technique was developed to measure the uptake of vitellogenin by oocytes *in vivo* and how this technique was then validated.

5.1. Development of a technique to measure the uptake of vitellogenin in the oocytes

The aim of the technique was to quantify the uptake of vitellogenin by oocytes *in vivo*. Determination of the uptake of vitellogenin by oocytes *in vitro* would not be appropriate as it is difficult to create an *in vitro* "ovarian" environment for the oocyte. Furthermore the uptake of

yolk precursor by oocytes may be under the control of other physiological parameters such as circulating levels of hormones and the position of the oocyte in the ovary.

To determine the uptake of vitellogenin in developing oocytes, the amount of vitellogenin entering the oocyte has to be quantified over a fixed period of time. As the determination of the amount of vitellogenin present in an oocyte at a given time requires the removal of this oocyte from the bird, it is impossible to determine, in a given oocyte, the increase in the amount of vitellogenin over a period of time.

To circumvent this problem, a technique involving the labelling of the vitellogenin protein with a radioactive marker was developed. As vitellogenin is a major transporter of ions and as the binding of ions to this protein does not impede its uptake by oocytes, mineral isotopes were considered suitable markers. Although vitellogenin is the main circulating calcium transporter in laying hens (Parsons and Combs, 1981) and up to 70% of calcium present in the yolk of domestic chickens is contained in a lipovitellin-phosvitin complex (Robinson *et al.*, 1979), the labile nature of plasma calcium in laying hen (Taylor and Dacke, 1984) precludes general use of this marker for estimation of vitellogenin. However, as vitellogenin contains 90% of the yolk zinc and as measurement of plasma zinc in laying hens has been demonstrated to provide an accurate technique for the estimation of the plasma vitellogenin concentration (Mitchell and Carlisle, 1991), radioactive zinc, ^{65}Zn (as $ZnCl_2$), was used to determine the rate of uptake of vitellogenin in the oocytes.

The technique involved labelling the vitellogenin with ^{65}Zn, isolating the vitellogenin-bound ^{65}Zn from the other plasma components, concentrating and purifying the vitellogenin-bound ^{65}Zn, injecting this preparation intravenously in birds and finally detecting and quantifying the vitellogenin-bound ^{65}Zn in the tissues and fluid samples. The use of ^{65}Zn as a marker for vitellogenin was possible as most of the circulating zinc is bound to this protein and because the vitellogenin-zinc complex is stable in the circulation.

5.1.1. Labelling of vitellogenin

A blood sample (4 ml) was obtained as described in Chapter 3 from a mature hen laying regularly and plasma was isolated. A volume of 2.5 ml of plasma was then mixed with 15 µl of $^{65}ZnCl_2$ which represented 48.6 µg of ^{65}Zn or 0.14 Mbq. The solution was then left for an hour at 41°C to allow the binding of the ^{65}Zn to proteins.

5.1.2. Isolation of vitellogenin-bound ^{65}Zn from other plasmatic components

The isolation of vitellogenin-bound ^{65}Zn was achieved by gel filtration chromatography in a 3.0 cm x 35.0 cm chromatography column (Sephadex, Pharmacia). The gel, composed of spherical Agarose beads with an operating range of 10 to 5,000 kDa, was prepared and calibrated as follows :

Preparation

1. Some chromatography column, or elution, buffer containing 0.9% NaCl, 20 mM Tris and 50 µg/ml PMSF adjusted to pH 7.4 with 20 mM HCl was prepared.
2. The gel was mixed with an equal volume of elution buffer in a beaker until no settled solution remained.
3. The solution was poured into the column with the bottom tap open. The buffer leaving the tap was collected. The gel was allowed to settle but not to dry out and more buffer was added until the column was almost full.
4. Some more gel was added until there was a definite layer of gel at the top. Then some clean fresh buffer was added to about 2 or 3 mm above the column of gel.
5. The bottom tap was closed after drips have stopped.
6. The cap was put on after making sure no air was trapped in the gel and tightened up.
7. The pump was connected to the column, the tap was opened and the buffer which exited was collected. The buffer was left to run through until the gel was solid and dry.
8. The fraction collector was then connected.

Calibration

Once the gel was ready, it was calibrated with markers of known molecular masses : Blue Dextran (2,000 kDa) and Bio-Rad Gel Filtration Standard, a lyophilised mixture of five molecular mass markers ranging from 1.35 to 670 kDa (Table 24). As Blue Dextran, myoglobin and vitamin B_{12} are coloured molecules, their elution volume was determined by visual observation of the eluted fractions. To confirm these observations and determine the elution volume of the other molecules, a protein assay was carried out on all the fractions (section 3.2.1.).

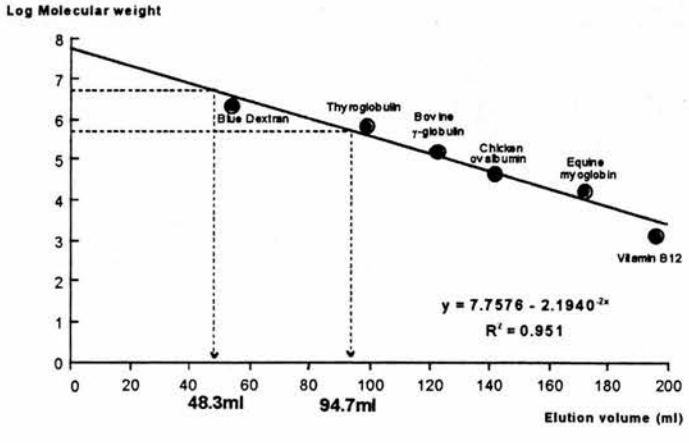
Table 24 : Markers in Bio-Rad filtration standard solution.

Markers	Molecular mass
Thyroglobulin (5.0 mg)	670 kDa
Bovine γ-globulin (5.0 mg)	158 kDa
Chicken ovalbumin (5.0 mg)	44 kDa
Equine myoglobin (2.5 mg)	17 kDa
Vitamin B_{12} (0.5 mg)	1.35 kDa

As the elution volume of the different markers is linearly related to the logarithm of their molecular masses, a linear regression between the elution volume and log molecular mass was established (Figure 19). As the upper operating molecular weight of the gel is 5,000 kDa, the void volume was calculated from the equation of the linear regression and found to be 48.3 ml and the expected elution volume of the vitellogenin (knowing its molecular mass : 480 kDa) was also determined and found to be 94.7 ml.

After the gel was calibrated, the next stage was to load plasma-bound ^{65}Zn onto the column. The aim of this stage was to determine whether the isolation of vitellogenin from the other components of the plasma which may also have been labelled (e.g. ovalbumin) was possible.

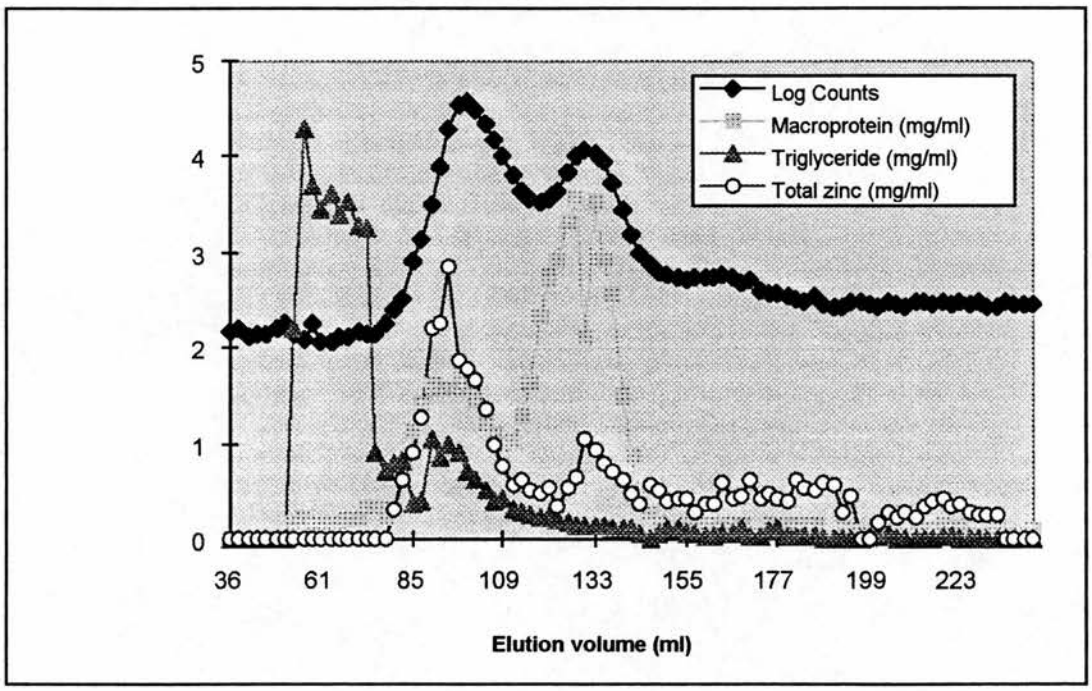
Figure 19 : Elution profile of Blue Dextran and Bio-Rad filtration standard solution (see text for details).



As ^{65}Zn is a high energy γ-emitter, vitellogenin-bound ^{65}Zn was easily detected with a γ-counter as it ran through the column. After no more radioactivity was detectable in the column, each fraction was assayed for protein, total zinc, triglyceride and radioactivity counts (determined

with a γ-counter over a period of 50 seconds). As expected, the plots of the radioactivity counts and protein (Figure 20) showed a peak for an elution volume between 93 and 106 ml which corresponded to the elution of the vitellogenin. Nearly 90% of the plasma triglyceride eluted approximately over the same time course as that of the predicted low density lipoprotein fraction. Another peak of radioactivity counts and protein appeared between 120 and 145 ml corresponding to the elution of ovalbumin. Fortunately vitellogenin and ovalbumin eluted at different times which rendered the isolation of vitellogenin possible.

Figure 20 : Profiles of radioactivity counts, total zinc, triglyceride and protein of plasma-bound ^{65}Zn.



5.1.3. Concentration and purification of vitellogenin-bound ^{65}Zn

The fractions containing vitellogenin-bound ^{65}Zn were then bulked to give a semi-purified vitellogenin solution. To reduce the volume of injection, the next stage was to concentrate this solution by centrifugal filtration. This technique is again based on the size of the molecules. Tubes (2.5 ml) containing filters with a molecular mass cut off point of 100 kDa were used. First, the tubes were rinsed to remove the glycerine. Approximately 2.5 ml of distilled water was added into each tube, the filters were placed on the water and the tubes were centrifuged at 1,500 g for 5 minutes. After discarding the water, the semi-purified vitellogenin solution was added into the tubes and the filters were placed on the solution. The tubes were centrifuged with gradually increasing speed (5 minutes at 2,000 g and 5 minutes at 4,000 g) to separate the

supernatant from the bottom of the tube. After removal of the supernatant, the purified and concentrated vitellogenin fractions were bulked to constitute the final vitellogenin-bound ^{65}Zn preparation.

The absence of free ^{65}Zn in this solution was confirmed by taking an aliquot of the solution and mixing it with an equal volume of dextran sulphate-magnesium chloride. The latter has been shown to selectively precipitate all the triglyceride-rich lipoproteins and the zinc bound to them but to leave free zinc in solution (Griffin and Mitchell, 1984) (see section 3.3.3.). The pellet was discarded and the remaining solution was assayed for protein concentration and radioactivity counts. As no protein and radioactivity were found in this solution, this assay demonstrated that all of the zinc and protein were precipitable and therefore that all the zinc was bound to the vitellogenin.

5.1.4. Conclusions

The labelling of natural vitellogenin present in high concentrations in fresh plasma of laying hens by incubation with ^{65}Zn proved extremely effective. Chromatography on an A5-agarose gel indicated that 60 to 70% of radioactive zinc binding occurred in a protein peak corresponding to the predicted elution volume and molecular weight of vitellogenin (Figure 20). The majority of the remaining ^{65}Zn binding eluted with a protein likely to be serum ovalbumin. The analysis of column eluents for triglyceride, zinc and protein confirmed separation of vitellogenin from VLDL and other potential zinc binding proteins and indicated protein rich fractions of high zinc content and ^{65}Zn specific activities.

5.2. Experiment 3

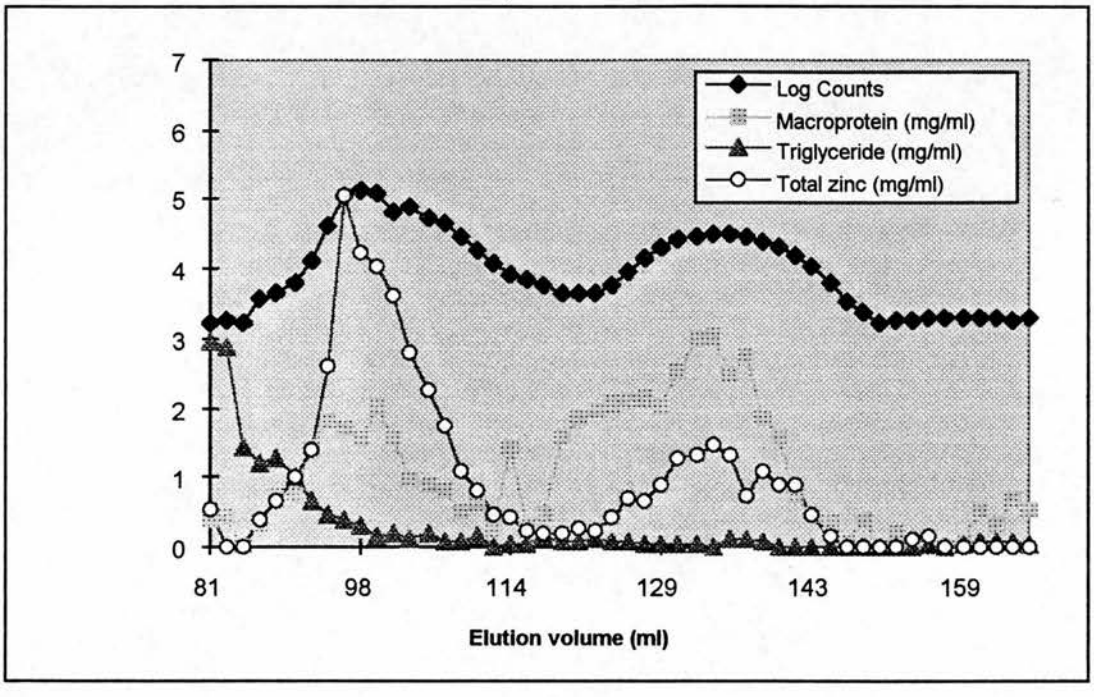
The objective of experiment 3 was to determine whether the technique developed in section 5.1. could be used to measure the uptake of vitellogenin by oocytes *in vivo*.

5.2.1. Preparation of vitellogenin-bound ^{65}Zn solution

To prepare the vitellogenin-bound ^{65}Zn solution, blood samples were collected from the brachial vein from two birds laying regularly. The plasma samples were prepared and bulked. A sample of approximately 4 ml of plasma was mixed with 50 µl of $^{65}ZnCl_2$ representing 162

μg of ^{65}Zn or 0.46 Mbq. The solution was then incubated for one hour at 41°C, run onto the column and the collected fractions of approximately 1.7 ml each were analysed for the radioactivity counts (per 50 seconds), total zinc, triglyceride and protein (Figure 21).

Figure 21 : Profiles of radioactivity counts, total zinc, triglyceride and protein of plasma-bound ^{65}Zn.



The fractions showing elution volumes between 92 and 105 ml were combined and concentrated by centrifugal filtration. After removal of the supernatant, the concentrated vitellogenin-bound ^{65}Zn was collected (1.2 ml) and radioactive counts, protein and zinc concentrations were determined (Table 25).

Table 25 : Composition of the injected solution.

Counts/s/ml	Protein concentration (mg/ml)	Vg-Zn concentration (μg/ml)	Protein / Vg-Zn concentration (mg protein / μg Zn)
7,600	13.45	9.72	1.38

5.2.2. Selection of the birds and experimental method

Four hens showing similar body weight (2,147 ± 71 g), producing eggs of similar weight (68.0 ± 0.9 g) and aged between 52 and 70 weeks were selected and injected intravenously through the brachial vein with 2.38 ± 0.41 μg of vitellogenin-bound ^{65}Zn (245 ± 42 μl or 1860 ± 320 counts per s per hen).

Assuming that the blood volume of a bird weighing 2,147 g is approximately 151 ml (Kotula and Helbacka, 1966), that the plasma volume is equal to 70% of the blood volume (Medway and Kare, 1959), and the plasma vitellogenin-bound zinc concentration, at 70 weeks of age, is approximately 3.4 μg per ml (Mitchell and Carlisle, 1991), the amount of endogenous vitellogenin-bound zinc in the circulation would be 360 μg. Therefore, the amount of vitellogenin-bound ^{65}Zn injected in the birds was very low (<1%) compared to the amount of vitellogenin naturally present in the circulation.

After a period of equilibration (2 minutes), a blood sample was taken from each bird from the brachial vein. The time at this stage was designated as T_0. Additional samples were also collected 15, 30, 60, 120 and 180 minutes after the first blood collection and these times were designated as T_{15}, T_{30}, T_{60}, T_{120} and T_{180} respectively. This was done to provide a time course for the clearance of radioactivity counts in the circulation. Furthermore, at each of the following times : T_{30}, T_{60}, T_{120} and T_{180}, one bird was killed by receiving a lethal dose of Pentobarbiturone (Rhone-Merieux, Southampton, UK) injected intravenously through the brachial vein and the liver, pectoral muscle and oocytes were collected for measurement of total counts.

Determinations of radioactivity in the liver and pectoral muscle were performed to assess to what extent vitellogenin-bound ^{65}Zn was taken up in organs other than the oocytes. Samples of approximately 1 g of liver and pectoral muscle and only the four largest oocytes (weighing more than 2.3 g) were collected. Each sample was weighed, homogenised in 5 ml of 0.9% saline (for liver and pectoral muscle tissues) and counted in a γ-counter (1277 Gamma Master, LKB-Pharmacia). To determine the radioactivity counts in the oocytes, each oocyte was counted separately. As the capacity of the γ-counter was not sufficient to accommodate entire oocytes, each oocyte was divided into two fractions: the membrane and attached yolk on one hand and the rest of the yolk on the other hand. These two fractions were counted individually and the values of their radioactivities were added to give the radioactivity in the entire oocyte.

5.2.3. Results : Radioactivity counts in the circulation

The radioactivity counts, and therefore the amount of ^{65}Zn, in the circulation decreased relatively quickly (Table 26). After 30 minutes, the radioactivity counts were reduced by 55% compared to their initial values (T_0). After two hours, 84% of the radioactivity was cleared and 93% was cleared after three hours (means based on the readings from the remaining birds). After a period of 1.30 h, a substantial proportion (approximately 80%) of the injected ^{65}Zn was therefore cleared from the circulation suggesting that an interval of 1.30 to 2 h after ^{65}Zn injection would therefore be sufficient to pick up differences, if any, in the proportion of vitellogenin-bound ^{65}Zn between different birds or treatments.

Table 26 : Blood radioactivity counts after injection (counts per s per ml of blood)

Bird	Time (*)					
	T_0	T_{15}	T_{30}	T_{60}	T_{120}	T_{180}
1	26.6	13.9 (52)	9.8 (37)	-	-	-
2	32.8	21.2 (65)	15.6 (48)	11.2 (34)	-	-
3	37.2	23.3 (63)	17.3 (47)	9.2 (25)	5.4 (15)	-
4	38.3	27.5 (72)	18.5 (48)	11.8 (31)	5.3 (14)	2.3 (6)
Mean	**33.7**	**21.5 (64)**	**15.3 (45)**	**10.7 (32)**	**5.3 (16)**	**2.3 (7)**
C.V.	**4.6**	**4.9**	**3.3**	**1.1**	**0.1**	**-**

(*) : Number in parentheses indicate % of T_0 value. C.V. : Coefficient of variation.

As can be seen in Table 26, the coefficient of variation between birds was relatively low. Therefore, at each time, the proportion of vitellogenin-bound ^{65}Zn cleared from circulation was very similar in all the birds. As both labelled and non-labelled vitellogenins are in equilibrium in the circulation, this also means that a similar **proportion** of total vitellogenin (including non-labelled vitellogenin) was cleared from the circulation at a given time in all the birds. However, this does not mean that a similar **amount** of total vitellogenin was cleared in all the birds. To relate the proportion to the amount, the concentration of vitellogenin in the plasma has to be determined.

5.2.4. Results : Radioactivity counts in the liver and pectoral muscle

The determination of the radioactivity counts found in the liver and in the pectoral muscle revealed that, three hours after the administration of vitellogenin-bound ^{65}Zn, detectable amounts of radioactivity were found in the liver but not in the pectoral muscle (Table 27). As the pectoral muscle has no involvement in the process of egg production and probably does not contain any vitellogenin receptors, the absence of radioactivity in this tissue was expected. The presence of vitellogenin-bound ^{65}Zn in the liver is probably due to the fact that a small fraction of circulating vitellogenin is taken up by the hepatocytes and recycled. Thirty minutes after the injection, radioactivity counts and thus vitellogenin-bound ^{65}Zn (section 5.1.) were already detectable in the liver and the counts increased with time. The accumulation of vitellogenin-bound ^{65}Zn in the liver may explain, at least in part, the clearance of vitellogenin-bound ^{65}Zn from the circulation.

Table 27 : Radioactivity counts in the liver and pectoral muscle at different times after injection (counts per s per g of tissue)

Bird	Time after injection	Liver	Pectoral muscle
1	T_{30}	4.9	0.1
2	T_{60}	6.3	0.0
3	T_{120}	7.3	0.0
4	T_{180}	9.0	0.2

5.2.5. Results : Radioactivity counts in the oocytes

The radioactivity counts detected in the oocytes, expressed per gram and per cm^2 of oocyte, are given in Table 28. The surface area of the oocyte was calculated from its volume which in turn was deduced from its weight.

As was observed in the liver, the greater the interval between vitellogenin-bound ^{65}Zn injection and killing of the bird, the higher the radioactivity counts in the oocytes. Furthermore, the results show that the counts per second and per gram of tissue were inversely related to the size of the oocyte, the uptake of vitellogenin per gram of tissue was greater in the smaller oocytes. Finally these results demonstrated that expression of the counts per unit weight (g) or per unit

surface (cm^2) led to the same relative variations between the birds.

Table 28 : Radioactivity counts in the oocytes at different times after injection (counts per s per g of tissue)

Bird	Parameters	Oocytes				
		1	2	3	4	Total
1 (killed at T_{30})	Weight (g)	14.33	10.97	5.65	4.77	**35.72**
	Surface (cm^2)	28.80	24.10	15.49	13.83	**82.22**
	Counts (s^{-1})	17.28	13.44	13.54	11.84	**56.10**
	Counts ($s^{-1}.g^{-1}$)	1.21	1.23	2.40	2.48	**1.57**
	Counts ($s^{-1}.cm^{-2}$)	0.60	0.56	0.87	0.86	**0.68**
2 (killed at T_{60})	Weight (g)	17.00	13.55	8.01	2.39	**40.95**
	Surface (cm^2)	32.27	27.75	19.54	8.73	**88.29**
	Counts (s^{-1})	20.20	19.82	13.66	12.06	**65.74**
	Counts ($s^{-1}.g^{-1}$)	1.19	1.46	1.71	5.05	**1.61**
	Counts ($s^{-1}.cm^{-2}$)	0.63	0.71	0.70	1.38	**0.74**
3 (killed at T_{120})	Weight (g)	16.52	12.82	8.79	4.76	**42.89**
	Surface (cm^2)	31.66	26.74	20.79	13.80	**92.99**
	Counts (s^{-1})	33.36	28.88	25.80	19.66	**107.70**
	Counts ($s^{-1}.g^{-1}$)	2.02	2.25	2.94	4.13	**2.51**
	Counts ($s^{-1}.cm^{-2}$)	1.05	1.08	1.24	1.42	**1.16**
4 (killed at T_{180})	Weight (g)	16.80	13.92	9.46	6.37	**46.55**
	Surface (cm^2)	32.02	28.25	21.84	16.77	**98.88**
	Counts (s^{-1})	25.22	27.10	36.74	28.84	**117.90**
	Counts ($s^{-1}.g^{-1}$)	1.50	1.95	3.88	4.53	**2.53**
	Counts ($s^{-1}.cm^{-2}$)	0.79	0.96	1.68	1.72	**1.19**

5.2.6. Discussion

The aim of experiment 3 was to determine whether it was possible to quantify the uptake of vitellogenin protein by oocytes *in vivo* by intravenously administrating birds with a solution of vitellogenin-bound ^{65}Zn. Determination of radioactivity in the plasma, liver and oocytes at

given periods of time after the injection with radiolabelled vitellogenin revealed it was possible to monitor the fate of this radiolabelled protein in the circulation and its target tissues. In the plasma, a substantial proportion (80%) of the radioactivity was cleared after 90 minutes and in the oocytes, the radioactivity increased constantly over the 180 minutes of the experiment. This technique was therefore appropriate to follow the evolution of the radioactivity in the plasma, liver and oocytes at different periods of time after the injection as well as the proportion of these counts compared to T_o or other sampling times.

However, to determine the actual amounts of vitellogenin cleared from plasma or taken up by the oocytes and to compare the uptake of vitellogenin between birds, determination of the radioactive counts alone is not sufficient. Indeed, a low radioactivity counts in the oocytes can be due to two reasons. Either the uptake of vitellogenin is low, or the concentration of total vitellogenin in the circulation (including the endogenous vitellogenin) is high. In the latter case, as the relative amount of vitellogenin-bound ^{65}Zn would be low compared to the total vitellogenin pool present in the bird, low amounts of vitellogenin-bound ^{65}Zn would be taken up in the oocytes even though the uptake may be high. Similarly, the comparison of the proportions of counts cleared in the plasma at fixed periods of time between different birds does not indicate how quickly total vitellogenin was cleared, as a low clearance of radioactive counts could be due to high amounts of endogenous vitellogenin in the bird.

To overcome this problem, one solution would be to administer the birds an amount of vitellogenin-bound ^{65}Zn which is directly proportional to the amount of endogenous vitellogenin present in the circulation at T_o. This, of course, implies collecting a sample of blood before the injection and determining its concentration in vitellogenin. However, as this determination takes a few hours, the concentration of vitellogenin in the circulation may have varied between the time the analysis is completed (and the bird injected) and the time the blood sample was collected. Injecting an amount of vitellogenin-bound ^{65}Zn which is directly proportional to the amount of endogenous vitellogenin is therefore very difficult and almost impossible. Secondly, this approach requires injecting the appropriate amount of vitellogenin-bound ^{65}Zn with extreme accuracy. This is also almost impossible as some of the injected vitellogenin-bound ^{65}Zn inevitably leaks outside the vein and is lost.

To establish a relation between the uptake of vitellogenin-bound ^{65}Zn to the uptake of total vitellogenin, another approach would be to collect a blood sample immediately before T_o and to determine its vitellogenin concentration later (once the experiment is finished). In the

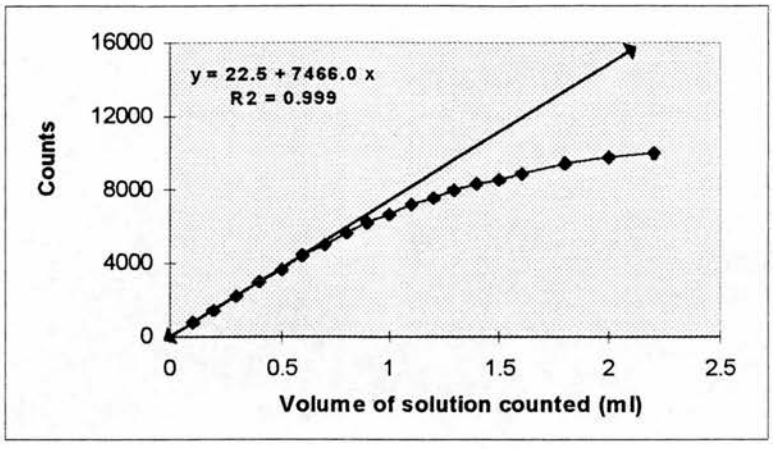
meantime, blood could be collected at fixed period of time after T_o and liver and oocytes could be collected at the end of the experiment. Then, once the experiment is finished, all these samples could be analysed for radioactivity counts and the plasma concentration of vitellogenin at T_o could be determined. The latter concentration could then be used to calculate the specific activity (S.A.) of the vitellogenin (counts at T_o per μg of Vg-Zn at T_o) in the plasma.

To determine the clearance of vitellogenin from the circulation, the value of the plasma concentration of vitellogenin at T_o could be used. By multiplying this value to the proportion of counts cleared in the circulation per unit time, the quantity of Vg-Zn cleared per unit time can be calculated. In the liver and oocytes, S.A. could be used to relate the counts observed to the uptake of Vg-Zn per gram of tissue. Then knowing the ratio between vitellogenin concentration and Vg-Zn concentration (from the composition of the injected solution, approximately 1.4), it would be possible to calculate, from the uptake or clearance of Vg-Zn (in μg per g of tissue or μg per ml of blood), the uptake or clearance of vitellogenin (in mg per g or mg per ml). Finally, determination of the volume of blood in the animal and of the weight of each organ will allow us to calculate the uptake or clearance of vitellogenin in the whole volume of blood or in a whole organ.

As determinations of Vg-Zn concentration in the plasma and radioactive counts in the plasma or tissues are the base for the calculations of clearance or uptake of vitellogenin, they had to be determined very accurately. A small trial was therefore carried out to test the efficiency of the γ-counter. Different volumes of a solution containing some vitellogenin-bound ^{65}Zn, ranging from 0.1 to 2.2 ml, were counted and the relationship between volume counted and radioactive counts is represented in Figure 22.

As demonstrated in this figure, the radioactivity (counts per s per ml) detected by the γ-counter were not directly proportional to the volume of solution counted. The greater the volume of solution, the lower the counts detected. To avoid having too big a discrepancy between counts detected by γ-counter and actual counts present in the solution, the sample volume should be equal to or less than 1 ml.

Figure 22 : Relationship between counts and volume of sample.



5.3. Conclusion

In this chapter, a technique for measuring vitellogenin uptake by oocytes was successfully developed (section 5.1.). Radioactive ^{65}Zn was used to label vitellogenin and a solution containing purified and concentrated vitellogenin-bound ^{65}Zn was prepared. An aliquot of this solution was then administered intravenously into birds and, over a period of three hours, the radioactivity counts found in the circulation, liver and oocytes were determined using a γ-counter.

These measurements allowed the determination of the proportion of counts cleared in the circulation or taken up by liver or oocytes per unit of time. However, these proportions were not sufficient to determine the uptake of vitellogenin by oocytes. To relate them to the actual amounts of vitellogenin cleared or taken up in these tissues, the plasma concentration of vitellogenin (as Vg-Zn) when the bird was injected the radiolabelled protein, or T_o, had to be measured. Therefore, in the next experiments, which objectives were to compare the uptake of vitellogenin in control and heat-stressed birds, and in non-treated and vitamin E-treated birds, the plasma concentration of vitellogenin at T_o was determined in addition to the counts found in circulation and oocytes.

Chapter 6

Effects of chronic heat stress and vitamin E on vitellogenin uptake by oocytes *in vivo*

In chapter 5, a technique was successfully developed to prepare a solution of pure and concentrated vitellogenin-bound ^{65}Zn. The intravenous administration of this solution to birds and the subsequent determination of the radioactivity found in their oocytes were shown to be appropriate for detecting uptake of vitellogenin-bound ^{65}Zn by oocytes. However, the validation of the technique on a few birds revealed that, in order to relate the uptake of vitellogenin-bound ^{65}Zn to the uptake of total vitellogenin, i.e. endogenous vitellogenin, plasma concentration of vitellogenin had to be determined prior to the injection. This was done in the experiments described in this chapter, in which the effects of heat stress and vitamin E were investigated on the uptake of vitellogenin by oocytes *in vivo*.

6.1. Experiment 4

The objective of experiment 4 was to investigate the effect of heat stress on the uptake of vitellogenin by oocytes *in vivo*.

6.1.1. Materials and methods

In Experiment 4, twenty four layers from a commercial line (I.S.A. Poultry services Peterborough, U.K.), genetically selected for high egg laying performance, were used. The age range extended from 52 to 70 weeks. Prior to the experiment, the birds were housed in a climate controlled room at 22°C, 50% Relative Humidity (R.H.) and kept in individual cages equipped with feeders and nippled-drinkers. Over a period of adaptation of eleven days, individual daily egg production and feed consumption were recorded. The birds received "*ad libitum*" access to a mash food (Table 29) and water.

From the egg production and feed intake data, twelve pairs of birds were constituted (2 birds per pair). Birds with similar egg production and body weight characteristics were paired. The birds were allocated into two groups designated control group (n=6) and heat stressed group (n=6). Both groups showed similar production characteristics and body weight at the start of the experiment (Table 30).

Table 29 : Composition and characteristics of the diet.

Composition	%	Characteristics	%
Wheat	36.00	Metabolisable energy (kcal/kg)	2,680
Barley	30.00	Crude proteins (%)	16.00
Soyabean meal (44% CP)	12.00	Fat (%)	3.77
Vegetable oil	1.50	Lysine (%)	0.73
Limestone	7.50	Methionine (%)	0.35
Salt	0.25	Methionine + cystine (%)	0.88
Lysine hydrochloride	0.09	Calcium (%)	3.57
Methionine	0.11	Available phosphorus (%)	0.40
Vitamin & mineral complex	0.55		

Table 30 : Characteristics of the two groups before the experimental period.

Control group					Heat stressed group				
Bird	**No of eggs produced (per day per hen)**	**Av. egg weight (g)**	**Body weight (g)**	**Feed intake (g)**	**Bird**	**No of eggs produced (per day per hen)**	**Av. egg weight (g)**	**Body weight (g)**	**Feed intake (g)**
14	0.818	62.37	1,860	113.5	**15**	0.818	61.15	1,970	116.8
22	1.000	72.70	1,880	140.1	**4**	0.818	72.12	1,960	104.8
11	1.091	64.65	1,920	112.5	**6**	0.909	64.79	1,940	137.8
19	0.727	64.23	2,170	109.3	**12**	0.909	62.68	2,090	125.0
5	0.818	64.68	2,200	101.3	**9**	0.727	68.57	2,110	107.9
20	0.818	77.00	2,280	129.7	**2**	1.000	75.57	2,310	119.7
Mean	**0.879 ± 0.125**	**67.60 ± 5.33**	**2,052 ± 169**	**117.7 ± 13.1**	**Mean**	**0.863 ± 0.087**	**67.48 ± 5.14**	**2,063 ± 128**	**118.7 ± 10.9**

At the beginning of the experiment, the birds were transferred into two temperature controlled chambers. The control group was housed under thermoneutral conditions (22°C, 50% R.H.) and the heat stressed group was exposed to chronic heat stress conditions (35°C, 60% R.H.). All the birds received a lighting pattern of 14 h light/10 h dark. Egg production and feed consumption were individually recorded for nine days (the experimental period). The birds received "*ad libitum*" access to water and to the same food as described in Table 29. At the end of the experimental period, the birds were injected with vitellogenin-bound ^{65}Zn as described in section 5.1. A sample of plasma (3 ml) was labelled with 620 µl of radioactive $^{65}ZnCl_2$ (5.70 Mbq), the solution was run onto the column and a solution of vitellogenin-bound ^{65}Zn was prepared. To increase the volume of this solution, another sample of plasma (3 ml) was then labelled with the same amount $^{65}ZnCl_2$, run onto the column and a second solution of vitellogenin-bound ^{65}Zn was prepared. The two solutions were then bulked, concentrated to give 25 ml of a concentrated vitellogenin-bound ^{65}Zn solution with a specific activity of 20.1 kBq/mg (Table 31).

Table 31 : Composition of the injected solution.

Counts (per s per ml)	Protein concentration (mg per ml)	Vg-Zn concentration (µg per ml)	Protein : Vg-Zn concentration (mg protein per µg Zn)
9,450	7.85	5.58	1.41

This solution was injected (1 ml per 1,000 g of body weight) into nine birds (five control birds and four heat stressed birds). The volume of solution injected represented 17,180 ± 2,160 counts per second per hen or 10.14 ± 1.28 mg of vitellogenin-bound ^{65}Zn per hen. To detect differences of uptake between the two groups, the amount of vitellogenin-bound ^{65}Zn injected per bird was higher than in Experiment 3. It represented approximately 3% of the total amount of vitellogenin present in a 2,000 g bird aged 70 weeks. Prior to the injection, number and weight of the eggs were monitored daily over a period of nine days and body weight of each bird was determined at the end of the nine days. Blood was sampled 1, 15, 30, 45, 60 and 75 minutes (T_1, T_{15}, T_{30}, T_{45}, T_{60} and T_{75}) after injection. After determination of blood radioactivity (samples of 1 ml to have a constant geometry for the counter, see section 5.2.6.) in each blood sample, the samples were centrifuged and plasma was collected. The plasma concentration of vitellogenin was then assayed in the samples collected at T_1. After the last blood sample collection (T_{75}), the birds were killed as described in section 5.2.2. The oocytes

and the liver were collected from each bird, weighed and counted for radioactivity.

For the liver and the oocytes, the radioactivity counts were also determined taking into account the geometry of the sample. After removal of the entire liver and the four largest oocytes from each bird, each organ was carefully weighed. The liver was then homogenised and two samples were taken (approximately 1 g) and each was counted twice. Each oocyte was also counted twice. For the bigger oocytes (>2 g), the membrane of the yolk was counted separately from the yolk and approximately 1 g of each sample was counted.

6.1.2. Effect of heat stress on egg production and Vg-Zn concentration

As was observed in Chapter 4, exposure to heat stress resulted in a marked decrease in all production parameters : -36% for egg number ($P<0.02$), -8% for mean egg weight (N.S.), -61% for feed consumption ($P<0.01$) and -18% for body weight ($P<0.01$) compared to the control group (Table 32).

Table 32 : Egg production and feed consumption during the experimental period.

Control group					Heat stressed group				
Bird	**No of eggs produced (per day per hen)**	**Av. egg weight (g)**	**Body weight (g)**	**Feed intake (g)**	**Bird**	**No of eggs produced (per day per hen)**	**Av. egg weight (g)**	**Body weight (g)**	**Feed intake (g)**
14	0.889	60.19	1,830	105.6	**15**	0.444	50.72	1,560	20.3
22	0.889	71.17	1,950	119.3	**4**	0.556	64.87	1,640	41.7
11	1.000	68.25	2,060	135.8	**6**	0.667	57.90	1,610	53.2
19	0.778	63.74	2,170	78.6	**12**	0.667	58.51	1,930	60.8
5	0.778	59.47	2,130	82.3	**9**	0.667	66.57	1,620	42.0
20	0.556	81.70	2,440	125.3	**2**	0.111	73.50	2,020	36.9
Mean	**0.815 ± 0.138 a**	**67.42 ± 7.61**	**2,097 ± 191 a**	**107.8 ± 21.3 a**	**Mean**	**0.519 ± 0.200 b**	**62.01 ± 7.28**	**1,730 ± 177 b**	**42.5 ± 12.7 b**

For a given parameter, the means indicated by different letters are statistically different ($P<0.05$).

Egg production and Vg-Zn concentration of the birds injected with vitellogenin-bound ^{65}Zn are presented in Table 33. In the heat stressed group, the plasma concentration of Vg-Zn was markedly reduced compared to the control group (-57%, $P<0.02$) and the number of eggs produced was reduced by a similar magnitude (-43%, $P<0.02$).

Table 33 : **Characteristics of the birds (egg production, body weight, plasma Vg-Zn concentration).**

Bird		No of eggs produced (per day per hen)	Mean egg weight (g)	Body weight (g)	Plasma Vg-Zn concentration (µg per ml)
Control	C.1	0.778	63.74	2,170	**2.26**
	C.2	1.000	68.25	2,060	**2.41**
	C.3	0.889	60.19	1,830	**3.00**
	C.4	0.778	61.62	1,490	**4.75**
	C.5	0.667	74.67	1,680	**2.35**
	Mean	0.822 ± 0.113 a	65.69 ± 5.25	1,846 ± 247	**2.95 ± 0.93 a**
Heat stress	H.S.1	0.111	73.50	2,020	**1.64**
	H.S.2	0.667	58.51	1,930	**1.85**
	H.S.3	0.667	66.57	1,620	**0.71**
	H.S.4	0.444	50.72	1,560	**0.86**
	Mean	0.472 ± 0.228 b	62.32 ± 8.55	1,782 ± 196	**1.26 ± 0.49 b**

For a given parameter, the means indicated by different letters are statistically different ($P<0.05$).

6.1.3. Results : Clearance of vitellogenin in the circulation

Radioactivity counts, determined in duplicate in each blood sample, are presented in Table 34.

Table 34 : **Radioactivity counts found in the blood at different times after injection (counts per s per ml of blood).**

Bird		Time					
		T_1	T_{15}	T_{30}	T_{45}	T_{60}	T_{75}
Control	C.1	199.8	99.1	67.1	50.9	39.2	32.7
	C.2	166.5	97.2	68.8	51.9	44.2	35.7
	C.3	127.2	71.9	49.9	39.8	35.5	28.2
	C.4	115.2	83.4	61.9	50.9	43.7	35.0
	C.5	73.7	47.7	33.4	26.5	20.0	16.9
Heat stress	H.S.1	150.4	92.3	63.3	46.4	36.8	31.9
	H.S.2	153.5	95.3	67.7	47.8	37.7	32.4
	H.S.3	183.0	47.2	19.7	11.4	9.4	7.1
	H.S.4	286.1	120.1	63.3	39.2	24.2	19.4

From these data, the relative proportion of counts which was present in the plasma at T_{15}, T_{30}, T_{45}, T_{60} and T_{75} was expressed as a percentage of the initial counts for each bird (computed from total counts detected one minute after injection, T_1) (Table 35).

Table 35 : Proportion of counts present at different times after injection (expressed as % of the initial counts).

Bird		Time					
		T_1	T_{15}	T_{30}	T_{45}	T_{60}	T_{75}
Control	C.1	100	49.6	33.6	25.5	19.6	16.4
	C.2	100	58.4	41.3	31.2	26.5	21.4
	C.3	100	56.5	39.2	31.3	27.9	22.2
	C.4	100	72.4	53.7	44.2	37.9	30.4
	C.5	100	64.7	45.3	36.0	27.1	22.9
Heat stress	H.S.1	100	61.4	42.1	30.9	24.5	21.2
	H.S.2	100	62.1	44.1	31.1	24.6	21.1
	H.S.3	100	25.8	10.8	6.2	5.1	3.9
	H.S.4	100	42.0	22.1	13.7	8.5	6.8

Knowing the concentration of Vg-Zn present in the plasma of each bird at the time of the injection and the proportion of vitellogenin-bound ^{65}Zn cleared from blood between each time interval, the amount of Vg-Zn which was cleared from plasma between the different time intervals can be calculated. For example, for the bird C.1, between T_1 and T_{15}, 50.4% of the initial vitellogenin-bound ^{65}Zn was cleared which implied that 2.26 µg x 0.504 = 1.14 µg of Vg-Zn was cleared per ml of plasma between T_1 and T_{15}. Between T_1 and T_{30}, the proportion of vitellogenin-bound ^{65}Zn cleared in the plasma was (1 - 0.336) = 66.4%. The amount of Vg-Zn cleared between T_1 and T_{30} was therefore 2.26 µg x 0.664 = 1.50 µg per ml of plasma. The amounts of Vg-Zn cleared from plasma between T_1 and the different sampling times are presented in Table 36.

Knowing the body weight of the birds (Table 33), the blood volume of these birds could be estimated by using the equation of Kotula and Helbacka (1966). As the plasma volume is about 70% of the blood volume (Medway and Kare, 1959), the plasma volume of each bird could also be estimated. As a result, the total amount of Vg-Zn and the equivalent amount of vitellogenin cleared per bird, obtained by multiplying the total amount of Vg-Zn by 1.4 (ratio

of Vg concentration to Vg-Zn concentration, Table 31), were calculated (Table 37).

Table 36 : Concentration of Vg-Zn cleared from the plasma at different times after injection (expressed in μg per ml of plasma).

Bird		Time					
		T_1	T_{15}	T_{30}	T_{45}	T_{60}	T_{75}
Control	C.1	0	1.14	1.50	1.68	1.82	1.89
	C.2	0	1.00	1.41	1.66	1.77	1.89
	C.3	0	1.30	1.82	2.06	2.16	2.33
	C.4	0	1.31	2.20	2.65	2.95	3.31
	C.5	0	0.83	1.29	1.50	1.71	1.81
Heat stress	H.S.1	0	0.63	0.95	1.13	1.24	1.29
	H.S.2	0	0.70	1.03	1.27	1.39	1.46
	H.S.3	0	0.53	0.63	0.67	0.67	0.68
	H.S.4	0	0.50	0.67	0.74	0.79	0.80

Table 37 : Total quantity of vitellogenin cleared at different times after injection (expressed in mg per bird).

Bird		Plasma volume (ml)	Time					
			T_1	T_{15}	T_{30}	T_{45}	T_{60}	T_{75}
Control	C.1	106	0	169	223	249	270	280
	C.2	104	0	146	205	242	258	275
	C.3	99	0	180	252	286	299	323
	C.4	93	0	171	286	345	384	431
	C.5	97	0	113	175	204	232	246
	Mean		**0**	**156**	**228**	**265**	**289**	**311**
Heat stress	H.S.1	102	0	90	136	161	177	184
	H.S.2	101	0	99	146	180	197	206
	H.S.3	95	0	70	84	89	89	90
	H.S.4	94	0	66	88	97	104	105
	Mean		**0**	**81**	**113**	**132**	**142**	**146**

At T_{75}, the amount of vitellogenin cleared from the circulation was significantly reduced in the heat stressed group compared to the control group (-53%, $P<0.01$). The magnitude of this

reduction is therefore similar to the decrease observed in the number of eggs produced (-43%, $P<0.02$) (see Table 33).

6.1.4. Results : Uptake of vitellogenin in the liver and oocytes

The uptake of Vg-Zn in the liver and oocytes was calculated using the Vg-Zn specific activity of each bird. Knowing the plasma Vg-Zn concentration at T_1, and therefore the Vg-Zn concentration in the blood (by multiplying by 70%) and the number of counts in the blood at T_1, the Vg-Zn specific activity was determined for each bird (Table 38).

Table 38 : Vitellogenin-bound zinc specific activity for each bird.

Bird		Initial counts (per s per ml of blood)	Vg-Zn concentration (µg per ml of blood)	Vg-Zn specific activity
Control	C.1	199.8	1.58	**126.5**
	C.2	166.5	1.69	**98.5**
	C.3	127.2	2.10	**60.6**
	C.4	115.2	3.32	**34.7**
	C.5	73.7	1.44	**51.2**
Heat stressed	H.S.1	150.4	1.15	**130.8**
	H.S.2	153.5	1.29	**119.0**
	H.S.3	183.0	0.50	**366.0**
	H.S.4	286.1	0.60	**476.8**

The uptake of vitellogenin in the liver (Table 39) and in the oocytes (Table 40) was then determined knowing the ratio of Vg concentration to Vg-Zn concentration. Follicles were considered separately and as a unit (hierarchy).

Table 39 : **Vitellogenin uptake by the liver within the 75 minutes of the experiment.**

Bird		Liver weight (g)	Counts (per s per g)	Vg-Zn concentration (µg per g of organ)	Vg-Zn concentration (µg per organ)	Equivalent mg of Vg per organ
Control	C.1	52.97	82.84	0.65	34.43	**48.55**
	C.2	54.89	94.34	0.96	52.69	**74.29**
	C.3	27.33	168.58	2.78	75.98	**107.13**
	C.4	48.45	73.02	2.10	101.74	**143.45**
	C.5	52.41	41.42	0.81	42.45	**59.85**
	Mean	**47.21 ± 10.16**				**86.65 ± 34.55 a**
Heat stressed	H.S.1	55.13	97.24	0.74	40.80	**57.53**
	H.S.2	48.89	101.82	0.86	42.05	**59.29**
	H.S.3	59.23	66.67	0.18	10.66	**15.03**
	H.S.4	24.34	205.04	0.43	10.47	**14.76**
	Mean	**46.90 ± 13.53**				**36.65 ± 21.77 b**

For a given parameter, the means indicated by different letters are statistically different ($P<0.05$).

In the liver and oocytes, the uptake of vitellogenin was significantly reduced in the heat stressed group compared to the control group (-58%, $P<0.05$ and -52%, N.S. respectively). These results are summarised in Table 41.

Table 40 : Vitellogenin uptake by the oocytes within the 75 minutes of the experiment.

Bird		Oocytes weight (g)	Counts (per s per g)	Vg-Zn concentration (µg per g of organ)	Vg-Zn concentration (µg per organ)	Equivalent mg of Vg per organ
C.1	1	12.98	21.10	0.17	2.21	**3.12**
	2	8.39	21.69	0.17	1.42	**2.00**
	3	3.43	56.41	0.44	1.51	**2.13**
	4	1.85	53.38	0.42	0.78	**1.10**
Total		**26.65**	**38.14**		**5.92**	**8.35**
C.2	1	14.16	19.43	0.20	2.83	**3.99**
	2	11.09	26.86	0.27	2.99	**4.22**
	3	8.53	29.97	0.30	2.56	**3.61**
	4	5.43	46.76	0.47	2.55	**3.60**
Total		**39.21**	**30.75**		**10.93**	**15.42**
C.3	1	15.00	3.52	0.06	0.90	**1.27**
	2	9.89	4.93	0.08	0.79	**1.11**
	3	4.50	14.83	0.24	1.08	**1.52**
	4	0.68	20.66	0.34	0.23	**0.32**
Total		**30.07**	**10.98**		**3.00**	**4.22**
C.4	1	15.80	12.45	0.36	5.69	**8.02**
	2	10.18	23.74	0.68	6.92	**9.76**
	3	4.31	45.66	1.32	5.69	**8.02**
	4	1.24	36.37	1.05	1.30	**1.83**
Total		**31.53**	**29.56**		**19.60**	**27.63**
C.5	1	15.93	8.83	0.17	2.71	**3.82**
	2	12.77	15.15	0.30	3.83	**5.40**
	3	8.96	15.38	0.30	2.69	**3.79**
	4	5.57	16.52	0.32	1.78	**2.51**
Total		**43.23**	**13.97**		**11.01**	**15.52**
H.S.1	1	9.48	31.14	0.24	2.28	**3.21**
	2	6.60	44.21	0.34	2.24	**3.16**
	3	2.89	85.25	0.65	1.88	**2.65**
	4	1.92	71.48	0.55	1.06	**1.49**
Total		**20.89**	**58.02**		**7.46**	**10.51**
H.S.2	1	15.34	17.26	0.15	2.30	**3.24**
	2	11.63	30.06	0.25	2.91	**4.10**
	3	7.75	30.46	0.26	2.01	**2.83**
	4	4.09	64.88	0.55	2.25	**3.17**
Total		**38.81**	**35.66**		**9.47**	**13.34**
H.S.3	1	11.54	20.20	0.06	0.69	**0.97**
	2	7.45	25.95	0.07	0.52	**0.73**
	3	4.90	38.94	0.11	0.54	**0.76**
	4	2.59	60.03	0.16	0.41	**0.58**
Total		**26.48**	**36.28**		**2.16**	**3.04**
H.S.4	1	4.13	5.08	0.01	0.04	**0.06**
	2	4.02	8.02	0.02	0.08	**0.11**
	3	3.02	8.55	0.02	0.06	**0.08**
	4	0.43	53.26	0.11	0.05	**0.07**
Total		**11.60**	**18.73**		**0.23**	**0.32**

Table 41 : **Comparison of vitellogenin uptake rates in the liver and in the oocytes in both control and heat stressed groups.**

Organ	Control birds	Heat stressed birds	Changes in heat stressed birds relative to control birds
Liver weight (g)	47.21 ± 10.16	46.90 ± 13.53	**- 1%**
Equivalent mg of Vg per liver	86.65 ± 34.55 a	36.65 ± 21.77 b	**- 58%**
Equivalent mg of Vg per g of liver	2.06 ± 1.18	0.78 ± 0.37	**- 62%**
Equivalent mg of Vg per g of liver per hour	1.65 ± 0.94	0.62 ± 0.30	**- 62%**
Hierarchy weight (g)	34.14 ± 6.13	24.44 ± 9.85	**- 28%**
Equivalent mg of Vg per hierarchy	14.23 ± 7.97	6.80 ± 5.31	**- 52%**
Equivalent mg of Vg per g of hierarchy	0.42 ± 0.25	0.24 ± 0.19	**- 43%**
Equivalent mg of Vg per g of hierarchy per hour	0.34 ± 0.20	0.19 ± 0.15	**- 42%**

For a given parameter, the means indicated by different letters are statistically different ($P<0.05$).

6.1.5. Discussion

Values in Table 41 allowed calculation of :

- The oocyte clearance determined as representing the volume of plasma totally cleared by the oocytes (µg of Vg-Zn per hierarchy per µg of Vg-Zn per ml of plasma) :

  Control birds = (14.23/1.4)/2.95 = 3.4 ml

  Heat stressed birds = (6.80/1.4)/1.26 = 3.8 ml

- The vitellogenin recovery : knowing the volume of plasma, the plasma Vg-Zn concentration at T_1, the ratio between Vg and Vg-Zn concentrations, the amount of vitellogenin cleared between T_1 and T_{75}, the quantity of vitellogenin present in the plasma at T_1 and T_{75} were determined. By adding the quantity of vitellogenin in the blood at T_{75}, in the liver and in the oocytes, the recovery of vitellogenin was calculated (Table 42) :

Table 42 : **Recovery of vitellogenin 75 minutes after the start of the experiment.**

Bird		Amount of Vg found in different tissues (mg per bird)				Recovery of Vg
		Plasma (T_1)	Plasma (T_{75})	Liver (T_{75})	Oocytes (T_{75})	
Control	C.1	335	55	49	8	**33%**
	C.2	351	76	74	15	**47%**
	C.3	416	93	107	4	**49%**
	C.4	618	187	143	28	**58%**
	C.5	319	73	60	16	**47%**
	Mean					**47%± 8%**
Heat stress	H.S.1	234	50	58	11	**51%**
	H.S.2	262	56	59	13	**49%**
	H.S.3	94	4	15	3	**23%**
	H.S.4	113	8	15	0	**20%**
	Mean					**36% ± 14%**

Despite the fact that detectable amounts of vitellogenin-bound ^{65}Zn were found in the liver and oocytes, the recovery of vitellogenin-bound ^{65}Zn was quite low in both groups of birds. This suggested that a noticeable fraction of the labelled vitellogenin must have gone in other organs than those involved in egg production and was therefore not detected. During heat stress, the recovery was even lower (-23%, N.S.) compared to thermoneutral conditions. This may be explained by an increase in the peripheral circulation during heat stress and may reflect an uptake or destruction of vitellogenin in cutaneous tissues.

The uptake of vitellogenin in the liver was reduced by 58% ($P<0.05$) in the heat stressed group compared to the control group. This could explain or be explained by the similar reduction in the circulating level of vitellogenin (-57%, $P<0.02$). The uptake of vitellogenin in the oocytes was also reduced during heat stress by 52% if we consider the total uptake by the hierarchy or by 43% if we consider the uptake per g of oocyte. The reductions of vitellogenin uptake by the liver and oocytes therefore occurred with a similar magnitude as the reduction of the concentration in vitellogenin in the plasma. The lower uptake of vitellogenin by the liver and oocytes may therefore have resulted from a lower availability of vitellogenin in the circulation but not from a direct effect of heat stress on the uptake process.

Experiment 4 therefore suggested that heat stress did not directly affect the uptake of vitellogenin. However, as this result does not exclude a possible effect of vitamin E, another experiment was carried out to investigate the effect of vitamin E on the uptake of vitellogenin by oocytes *in vivo*.

6.2. Experiment 5

The objective of experiment 5 was to investigate the effect of vitamin E on the uptake of vitellogenin by oocytes *in vivo*.

6.2.1. Materials and methods

In Experiment 5, twenty four layers from the same strain and age as those used in experiment 4 were selected, kept a climate controlled room at 22°C, 50% Relative Humidity (R.H.), and fed a similar diet as this described in experiment 4 (Table 29). Over a period of adaptation (35 days), individual egg production and feed consumption were recorded to allocate the birds into pairs and form two groups designated the control group (n=12) and the vitamin E supplemented group (n=12). Both groups showed similar production characteristics and body weight at the start of the experiment (Table 43).

Table 43 : Characteristics of the two groups of birds before the experiment.

	Control group (n=12)	**Vitamin E group (n=12)**
No of eggs produced (per day per hen)	0.779 ± 0.275	0.798 ± 0.257
Mean egg weight (g)	62.04 ± 5.77	62.62 ± 4.11
Feed intake (g)	107.72 ± 21.02	108.89 ± 14.99
Body weight (g)	1949 ± 87	1961 ± 138

After allocating the birds into pairs and distributing these pairs in a climate-controlled room (22°C, 50% R.H.), birds were fed the experimental diets for 15 days. These diets showed the same basal composition and characteristics as the diet distributed during the adaptation period but differed in their vitamin E content : 10 and 250 mg α-tocopherol per kg diet. During this

period, egg production, feed intake and body weight were not affected by the vitamin E treatment (Table 44).

Table 44 : Characteristics of the two groups after the administration of the experimental regimes.

	Control group (n=12)	Vitamin E group (n=12)
No of eggs produced (per day per hen)	0.828 ± 0.268	0.812 ± 0.255
Mean egg weight (g)	63.64 ± 5.04	65.19 ± 3.27
Feed intake (g)	130.40 ± 15.46	127.36 ± 20.30
Body weight (g)	2022 ± 121	2023 ± 146

At the beginning of the experiment, the ambient temperature in the room was increased from 22°C to 35°C and the relative humidity was increased from 50% to 60%. These conditions were applied to the birds for eight days, during which time egg production and feed consumption of individual birds were recorded.

After the 8-day exposure to heat stress, a purified solution of labelled vitellogenin was prepared as described in Chapter 5. A sample of plasma (3 ml) was collected and labelled with 490 µl of radioactive $^{65}ZnCl_2$ (4.5 Mbq). The solution was then run onto the column and a volume of 25 ml of vitellogenin-bound ^{65}Zn was prepared as described previously. Its specific activity was of 13.8 kBq per mg (Table 45).

Table 45 : Composition of the injected solution.

Counts (per s per ml)	Protein concentration (mg per ml)	Vg-Zn concentration (µg per ml)	Protein : Vg-Zn concentration (mg protein per µg Zn)
3,660	4.44	3.19	1.39

After the period of heat stress, this solution was injected (1.7 ml per 1,000 g of body weight) into seven birds (four control and three vitamin E-supplemented birds). Blood samples were taken at the following intervals after injection : 1.30, 3, 6, 12, 24, 48 and 96 minutes (or $T_{1.30}$, T_3, T_6, T_{12}, T_{24}, T_{48} and T_{96}). After counting blood radioactivity (samples of 0.4 ml), the

samples were centrifuged and the plasma fractions collected at $T_{1.30}$ and T_{96} were assayed for the plasma total zinc and Vg-Zn concentrations. After T_{96}, the birds were killed, the oocytes and the liver were collected, weighed and counted for radioactivity.

6.2.2. Effect of vitamin E on egg production and Vg-Zn concentration

Egg production, food intake and body weight results are given in Table 46.

Table 46 : Egg production and feed consumption of the birds after the heat stress.

	Control group (n=12)	Vitamin E group (n=12)
No of eggs produced (per day per hen)	0.760 ± 0.267	0.865 ± 0.267
Mean egg weight (g)	60.59 ± 4.65	63.52 ± 2.80
Feed intake (g)	64.71 ± 21.33	67.19 ± 17.28
Body weight (g)	1866 ± 89	1817 ± 129

During exposure to heat stress, both the number and the weight of eggs were lower in the control group compared to the group supplemented with 250 mg of vitamin E per kg diet : -10.5% for egg production (N.S.) and -2.9 g for mean egg weight ($P<0.08$). The fact that these differences failed to reach significance may be explained by the relatively small number of birds used in this experiment and the short duration of the stress period. The characteristics of the birds which were injected with vitellogenin-bound ^{65}Zn are given in Table 47.

Total egg mass (No of eggs x mean egg weight) was significantly improved during heat stress in the vitamin E-supplemented birds compared to controls (+15.4%, $P<0.01$). However, no difference in plasma Vg-Zn concentration was apparent between the two groups. This was not consistent with the observations of Utomo *et al.* (1994) showing that vitamin E increased the plasma concentration of vitellogenin in heat stressed compared to control birds.

Table 47 : **Characteristics of the birds (egg production, body weight, plasma Vg-Zn concentration).**

Bird		No of eggs produced (per day per hen)	Mean egg weight (g)	Body weight (g)	Plasma Vg-Zn concentration (µg per ml) $T_{1.30}$	T_{96}
Control	C.1	0.875	59.30	1,770	2.70	2.07
	C.2	0.875	61.41	2,020	2.02	1.94
	C.3	0.875	61.11	1,850	3.04	2.37
	C.4	0.875	65.13	1,810	2.10	1.31
	Mean	**0.875 ± 0.000**	**61.74 ± 2.12**	**1,862 ± 95**	**2.46 ± 0.42**	**1.92 ± 0.39**
Vitamin E	V.1	1.000	63.26	1,720	2.62	2.05
	V.2	1.000	61.80	1,850	2.27	1.91
	V.3	1.000	61.93	1,650	1.79	1.36
	Mean	**1.000 ± 0.000**	**62.33 ± 0.66**	**1,740 ± 83**	**2.23 ± 0.34**	**1.77 ± 0.30**

For a given parameter, the means indicated by different letters are statistically different ($P<0.05$).

6.2.3. Results : Clearance of vitellogenin in the circulation

The radioactivity counts were determined in triplicate in each blood sample and the mean values are given in Table 48.

Table 48 : **Radioactivity counts found in the blood at different times after injection (counts per s per ml of blood).**

Bird		Time						
		$T_{1.30}$	T_3	T_6	T_{12}	T_{24}	T_{48}	T_{96}
Control	C.1	70.8	61.1	51.3	41.6	31.8	22.1	12.3
	C.2	79.4	67.5	55.5	43.5	31.6	19.6	7.6
	C.3	75.0	65.0	55.1	45.3	35.3	25.5	15.5
	C.4	43.5	37.2	31.0	24.8	18.5	12.3	6.1
Vitamin E	V.1	87.4	74.5	61.7	48.7	35.8	23.0	10.1
	V.2	87.5	74.1	60.7	47.2	33.9	20.4	7.0
	V.3	73.4	62.1	50.7	39.4	28.1	16.7	5.4

From these data, the relative proportion of counts present in the plasma at T_3, T_6, T_{12}, T_{24}, T_{48} and T_{96} was expressed as a percentage of the initial counts for each bird (computed from total

counts detected at $T_{1.30}$) and the amount of Vg-Zn cleared from the plasma between $T_{1.30}$ and the different sampling times were then calculated as described in section 6.1.3. (Table 49).

Table 49 : Concentration of Vg-Zn cleared from the plasma at different times after injection (expressed in µg per ml of plasma).

Bird		Time						
		$T_{1.30}$	T_3	T_6	T_{12}	T_{24}	T_{48}	T_{96}
Control	C.1	0	0.37	0.74	1.11	1.45	1.86	2.23
	C.2	0	0.30	0.61	0.91	1.22	1.52	1.83
	C.3	0	0.40	0.81	1.20	1.61	2.01	2.41
	C.4	0	0.30	0.60	0.90	1.21	1.51	1.81
Vitamin E	V.1	0	0.39	0.77	1.16	1.55	1.93	2.32
	V.2	0	0.35	0.69	1.05	1.39	1.74	2.09
	V.3	0	0.28	0.55	0.83	1.10	1.38	1.66

The total amount of vitellogenin cleared per bird was then calculated as described in section 6.1.3. (Table 50).

Table 50 : Total quantity of vitellogenin cleared at different times after injection (expressed in mg per bird).

Bird		Plasma volume (ml)	Time						
			$T_{1.30}$	T_3	T_6	T_{12}	T_{24}	T_{48}	T_{96}
Control	C.1	98	0	51	102	152	199	255	306
	C.2	102	0	43	87	130	174	217	261
	C.3	99	0	55	112	166	223	279	334
	C.4	99	0	42	83	125	168	209	251
	Mean		**0**	**48**	**96**	**143**	**191**	**240**	**288**
Vitamin E	V.1	97	0	53	105	158	210	262	315
	V.2	99	0	49	96	146	193	241	290
	V.3	96	0	38	74	112	148	185	223
	Mean		**0**	**47**	**92**	**139**	**184**	**229**	**276**

At T_{96}, no difference in the amount of vitellogenin cleared from the circulation appeared between the control and vitamin E-supplemented groups.

6.2.4. Results : Uptake of vitellogenin in the liver and oocytes

The Vg-Zn specific activity was determined as described in section 6.1.4. (Table 51).

Table 51 : Vitellogenin-bound zinc specific activity for each bird.

Bird		Initial counts (per s per ml of blood)	Vg-Zn concentration (µg per ml of blood)	Vg-Zn specific activity
Control	C.1	101.2	1.89	**53.5**
	C.2	113.4	1.41	**80.4**
	C.3	107.1	2.13	**50.3**
	C.4	62.2	1.47	**42.3**
Vitamin E	V.1	124.9	1.83	**68.3**
	V.2	125.0	1.59	**78.6**
	V.3	104.8	1.25	**83.8**

The vitellogenin uptake in the liver (Table 52) and in the oocytes (Table 53) was then determined as described in section 6.1.4.

Table 52 : Vitellogenin uptake by the liver within the 96 minutes of experiment.

Bird		Liver weight (g)	Counts (per s per g)	Vg-Zn concentration (µg per g of organ)	Vg-Zn concentration (µg per organ)	Equivalent mg of Vg per organ
Control	C.1	42.38	54.55	1.02	43.23	**58.70**
	C.2	50.50	59.90	0.75	37.87	**52.64**
	C.3	43.47	55.15	1.10	47.82	**66.47**
	C.4	51.33	31.00	0.73	37.47	**52.08**
	Mean	**46.92 ± 4.02 a**				**57.47 ± 5.81**
Vitamin E	V.1	40.69	62.40	0.91	37.03	**51.47**
	V.2	36.36	79.75	1.01	36.72	**51.04**
	V.3	39.65	83.55	1.00	39.65	**55.11**
	Mean	**38.90 ± 1.85 b**				**52.54 ± 1.83**

For a given parameter, the means indicated by different letters are statistically different ($P<0.05$).

Table 53 : **Vitellogenin uptake by the oocytes within the 96 minutes of experiment.**

Bird		Oocytes weight (g)	Counts (per s per g)	Vg-Zn concentration (µg per g of organ)	Vg-Zn concentration (µg per organ)	Equivalent mg of Vg per organ
C.1	1	13.35	8.79	0.16	2.14	**2.97**
	2	8.96	9.21	0.17	1.52	**2.11**
	3	4.63	16.61	0.31	1.44	**2.00**
	4	1.84	29.08	0.54	0.99	**1.38**
Total		**28.78**	**15.92**		**52.79**	**8.46**
C.2	1	12.83	10.95	0.14	1.80	**2.50**
	2	9.27	7.64	0.10	0.93	**1.29**
	3	5.67	11.93	0.15	0.85	**1.18**
	4	3.12	14.04	0.17	0.53	**0.74**
Total		**30.89**	**11.14**		**4.11**	**5.71**
C.3	1	9.58	9.59	0.19	1.82	**2.53**
	2	5.49	16.15	0.32	1.76	**2.44**
	3	2.37	32.56	0.65	1.54	**2.14**
	4	0.64	32.11	0.64	0.41	**0.57**
Total		**18.08**	**22.60**		**5.53**	**7.68**
C.4	1	12.54	6.74	0.16	2.01	**2.79**
	2	9.94	7.04	0.17	1.69	**2.35**
	3	6.92	10.54	0.25	1.73	**2.40**
	4	3.25	20.84	0.49	1.59	**2.21**
Total		**32.65**	**11.29**		**7.02**	**9.75**
V.1	1	9.60	12.72	0.19	1.82	**2.53**
	2	5.88	22.13	0.32	1.88	**2.61**
	3	1.47	47.49	0.69	1.01	**1.40**
	4	0.72	37.36	0.55	0.40	**0.56**
Total		**17.67**	**29.92**		**5.11**	**7.10**
V.2	1	9.88	15.67	0.20	1.98	**2.75**
	2	6.86	20.14	0.26	1.78	**2.47**
	3	3.14	35.96	0.46	1.44	**2.00**
	4	1.39	32.50	0.41	0.57	**0.79**
Total		**21.27**	**26.07**		**5.77**	**8.01**
V.3	1	11.74	6.81	0.08	0.94	**1.31**
	2	6.51	15.79	0.19	1.24	**1.72**
	3	2.72	32.10	0.38	1.04	**1.45**
	4	0.83	20.90	0.25	0.21	**0.29**
Total		**21.80**	**18.90**		**3.43**	**4.77**

The uptake of vitellogenin by the liver and by the oocytes was similar in both control and supplemented groups. These results are summarised in Table 54.

Table 54 : **Comparison of vitellogenin uptake rates in the liver and in the oocytes in both control and vitamin E-supplemented groups.**

Organ	Control birds	Vitamin E birds	Changes in Vit. E birds relative to control birds
Liver weight (g)	46.92 ± 4.02 a	38.90 ± 1.85 b	**- 17 %**
Equivalent mg of Vg per liver	57.47 ± 5.81	52.54 ± 1.83	**- 9%**
Equivalent mg of Vg per g of liver	1.24 ± 0.22	1.35 ± 0.06	**+ 9%**
Equivalent mg of Vg per g of liver per hour	0.77 ± 0.14	0.84 ± 0.04	**+ 9%**
Hierarchy weight (g)	27.60 ± 5.66	20.25 ± 1.83	**- 27%**
Equivalent mg of Vg per hierarchy	7.90 ± 1.46	6.61 ± 1.39	**- 16%**
Equivalent mg of Vg per g of hierarchy	0.30 ± 0.08	0.33 ± 0.08	**+ 10%**
Equivalent mg of Vg per g of hierarchy per hour	0.19 ± 0.05	0.21 ± 0.05	**+ 11%**

For a given parameter, the means indicated by different letters are statistically different ($P<0.05$).

6.2.5. Discussion

The uptake rate of vitellogenin in the liver and oocytes was determined by using the radioactivity measurements in these two tissues. These determinations are therefore based on the assumption that, as a result from the high affinity zinc binding property of vitellogenin, all the ^{65}Zn remained bound to the vitellogenin over the duration of the experiment. Values in Table 54 allowed the determination of :

- The oocyte clearance determined as representing the volume of plasma totally cleared by the oocytes (µg of Vg-Zn per hierarchy per µg of Vg-Zn per ml of plasma) :

  Control birds = (7.90/1.4)/2.46 = 2.3 ml

  Vitamin E birds = (6.61/1.4)/2.23 = 2.1 ml

- The vitellogenin recovery : knowing the volume of plasma, the Vg-Zn concentration at $T_{1.30}$, the ratio between Vg and Vg-Zn concentrations, the amount of vitellogenin cleared between

$T_{1.30}$ and T_{96}, the quantity of vitellogenin present in the plasma at $T_{1.30}$ and T_{96} were determined. By adding the quantity of vitellogenin in the blood at T_{96}, in the liver and in the oocytes, the recovery of vitellogenin was calculated (Table 55) :

Table 55 : Recovery of vitellogenin 96 minutes after the start of the experiment.

Bird		Amount of Vg found in different tissues (mg per bird)				Recovery of Vg
		Plasma (T_1)	Plasma (T_{75})	Liver (T_{75})	Oocytes (T_{75})	
Control	C.1	370	64	59	8	**36%**
	C.2	288	27	53	6	**30%**
	C.3	421	87	66	8	**39%**
	C.4	291	40	52	10	**35%**
	Mean					**35%± 3%**
Vitamin E	V.1	356	41	51	7	**28%**
	V.2	315	25	51	8	**27%**
	V.3	241	18	55	5	**32%**
	Mean					**29% ± 2%**

The uptake of vitellogenin in the liver was decreased in the vitamin E group by 9% (N.S.) compared to the control group which could explain or be explained by the similar reduction in the circulating level of vitellogenin (-9%, N.S.). The uptake rate of vitellogenin in the oocytes was also reduced in the vitamin E group compared to the control group by a proportion of 16% if we consider the total uptake by the hierarchy (N.S.) or increased by 10% if we consider the uptake by g of oocyte. This reduction cannot explain the increase in the total mass of eggs produced (+15%, $P<0.01$).

As a result, the absence of effect of the vitamin E treatment on the uptake of vitellogenin in the liver and oocytes was consistent with the absence of effect of vitamin E on the plasma concentration of vitellogenin found in this experiment. This experiment therefore demonstrated, even though the amounts of vitellogenin recovered in the groups of birds was relatively low, that the uptake of vitellogenin by oocytes was not affected by the vitamin E treatment.

6.3. Conclusion

Experiment 4 was carried out to investigate the effect of heat stress on the uptake of vitellogenin by oocytes. Two groups of birds were therefore used and exposed to different climatic conditions during a period of nine days. One group of birds was kept under thermoneutral conditions (22°C, 50% R.H.) and another group was exposed to heat stress conditions (35°C, 60% R.H.). In this experiment, the uptake of vitellogenin by liver and oocytes was reduced in the heat stressed group compared to the thermoneutral group. Over a period of 75 minutes, the uptake of vitellogenin dropped from 87 mg per liver in the thermoneutral group to 37 mg in the heat stressed group, which, per g of liver and per hour, represented a drop from 1.65 to 0.62 mg per g per h. In the oocytes, the uptake of vitellogenin was also reduced by the heat exposure and represented a drop from 0.34 to 0.19 mg per g per h. The magnitude of the differences between thermoneutral and heat stressed birds (-62%, $P<0.08$ and -42%, N.S.) were similar to the reduction in the vitellogenin concentration in the circulation (-57%, $P<0.02$), and in the number of eggs produced (-43%, $P<0.02$).

Experiment 5 was carried out to investigate the effect of vitamin E on the uptake of vitellogenin by oocytes in heat-stressed birds. Two groups of birds, kept under heat stress conditions (35°C, 60% R.H.) during eight days, were fed different levels of vitamin E : 10 or 250 mg α-tocopherol per kg of diet. In this experiment, the uptake of vitellogenin by liver and oocytes was not affected by the vitamin E treatment. Over a period of 96 minutes, the uptake of vitellogenin was of 57 mg per liver in the non-supplemented group and of 53 mg in the supplemented group, which, per g of liver and per hour, represented an uptake of 0.77 and 0.84 mg per g per h respectively. In the oocytes, the uptake of vitellogenin was also unaffected by the vitamin E treatment and represented values of 0.19 and 0.21 mg per g per h respectively. The absence of significant differences between the two groups was therefore in accordance with the absence of differences in the vitellogenin concentration in the circulation (-9%, N.S.) and in the number of eggs produced (+14%, N.S.).

In both experiments, the magnitude of the difference of uptake of vitellogenin by oocytes between the tested groups were similar to the magnitude of the differences of vitellogenin concentration in the circulation. As a result, neither heat stress nor vitamin E supplementation seemed to have had any additional effect on the uptake of vitellogenin by oocytes than the one imposed by the decrease in vitellogenin concentration in the circulation. Any direct effect of heat stress or vitamin E on the uptake of vitellogenin by oocytes can therefore be discarded.

Chapter 7

Effects of chronic heat stress and vitamin E on circulating 17β-oestradiol concentration

Previous experiments demonstrated that, although vitamin E supplementation improved egg production during and after heat stress, it did not cause this effect through a stimulation of uptake of egg yolk precursor, vitellogenin, by oocytes. Indeed, in both experiments 4 and 5, the uptake of vitellogenin in heat stressed and vitamin E-supplemented birds appeared to be closely related to the concentration of vitellogenin in the plasma of these birds. Therefore vitamin E could alleviate the detrimental effects of heat stress by increasing the circulating concentration of vitellogenin rather than by improving its uptake in oocytes. As the synthesis of vitellogenin by the liver is regulated by the reproductive hormones, and more particularly oestrogens, the objective of the studies described in this chapter was to determine whether exposure to heat stress and supplementation with vitamin E affected the concentration of circulating 17β-oestradiol and whether vitamin E improved egg production by increasing the concentration of this hormone during heat stress.

7.1. Methods for the determination of circulating 17β-oestradiol concentration

The earliest assays for oestrogens were biological assays including that described by Allen and Doisey (1923) which depends on alterations in the appearance of the vaginal smear of the castrate mouse and that of Astwood (1938) which depends on the increase in uterine weight of immature rats. Later on, oestrogens were quantified by gas-liquid chromatography with electron capture detectors (Attal and Eik-Nes, 1968), by colorimetric or fluorometric determination, or indirectly by measuring the rate of reaction of certain oestrogen-sensitive enzymes (for review, see O'Donnell and Preedy, 1967). A double isotope derivative method for oestrogen measurement was also developed (Svendsen and Sorensen, 1964; Baird and Guevara, 1969). Better sensitivity (low nanogram range) could be achieved by using fluorometry or the double isotope derivative method; the lower limits of other methods are 0.2

to 0.3 μg. However, all of the above methods were limited by their general lack of sensitivity and the necessity for extensive purification of the samples prior to quantification.

Best sensitivity was achieved with competitive protein-binding assays. These have been carried out using a preparation of receptor protein present in the supernatant fraction of homogenised uteri of certain animals (Korenman *et al.*, 1970). Oestradiol was extracted from the biological fluid and then mixed with a standard solution of rabbit uterine cytosol and a suitable ^{14}C- or tritium-labelled authentic oestradiol. The conditions were chosen so that the receptor protein of the solution was just saturated with the radioactive substance. Since the portion of the oestradiol pool bound to the receptor protein was in dynamic equilibrium with the unbound portion, the oestradiol in the sample competed for binding sites with the radioactive standard and will displace a fraction of the latter. Thus the measured percentage of tracer bound to the receptor protein fell proportionally. By determining the amount of radioactivity either in the protein-bound or in the free fraction, the displacement of the marker oestradiol could be quantified. These assays were developed for several other plasmatic steroids, including the corticosteroids, progesterone and testosterone (Murphy, 1970). Radioimmunoassays in which antibodies were used as binding proteins have also been developed (Ábrahám, 1969). Both the protein-binding and radioimmunoassay methods were far more sensitive than any of those techniques previously mentioned and, thus, provided sufficient sensitivity to measure the low picogram quantities of oestrogen present in 2 to 3 ml of the plasma sample.

These significant advantages, including high sensitivity and high specificity, explained the widespread use of protein-binding assays and radioimmunoassays compared to many other quantitative assays, particularly for substances that are otherwise measured by pharmacological assays.

Although both the binding protein and the antiserum used for oestrogen determinations were highly specific for oestrogen, some preliminary isolation was necessary when either method was used for individual evaluation of oestradiol level. Both methods were similar in speed and ease of application. However, unlike uterine cytosol preparations, oestrogen antiserum did not bind synthetic oestrogen. Another advantage to measuring oestrogen by radioimmunoassay was the stability of the antiserum and the ease with which it could be obtained. The antiserum could be purchased or prepared by immunisation of rabbits or sheep with a commercially available oestrogen-bovine serum albumin conjugate. In either case, large quantities of the antiserum were stored frozen as a concentrate or as a lyophilised preparation for extended

periods of time. Repeated use of the same antiserum was advantageous since it permitted assay reproducibility over a number of years.

7.2. Methodology for radioimmunoassays

7.2.1. Description of the techniques

7.2.1.1. Purification

As the plasma of a laying hen contains large amount of lipid, an initial purification step was required to remove the lipids. Two methods of purification of oestradiol were tested. A first method consisted of a solvent (hexane:ethyl acetate) extraction. A second method consisted of an affinity chromatography extraction.

The first method was performed as described in Pantex extraction ^{125}I oestradiol kit (Catalogue n° 047). A sample of plasma was mixed with a known volume of solvent (hexane:ethyl acetate, 3:2). After shaking the mix for one minute, the upper layer was removed. The solvent was then evaporated in a vacuum oven and, when dry, the sample was reconstituted with diluent.

The second purification method was performed with antibodies that described by Glencross *et al.* (1981). The high specificity of these antibodies made them extremely valuable tools for rapid, selective purification of antigens. In principle, the antibody immobilised on a column support was used to selectively adsorb antigen from a mixture containing many other proteins (Livingston, 1974; Dalchau and Fabre, 1982). The other proteins, for which the antibody had no affinity, were washed away, and the purified antigen was then eluted from the immunoadsorbent.

7.2.1.2. Quantitation

Radioimmunoassay is often described in terms of the competition between a radiolabelled antigen (Ag*) and its unlabelled counterpart (Ag) for binding to a limited amount of specific antibody (Ab) (Felber, 1975). In most radioimmunoassay, the reaction is allowed to proceed to equilibrium and thus, can be represented by the following relation :

$$Ag^* + Ag + 2Ab \rightleftharpoons Ag^*Ab + AgAb$$

The concentration of the antibody is limited such that the labelled antigen, although present in trace amount, is in relative excess over the antibody. Thus, even in the absence of unlabelled antigen, only some of the radioactive antigen will be associated with the antigen-antibody complex while the remainder will be free in solution. In the radioimmunoassay, the total amounts of antibody and radiolabelled antigen are kept constant. The presence of unlabelled antigen will result in less of the labelled species being able to bind to the antibody. The greater the amount of unlabelled antigen (Ag) present, the lower will be the amount of radiolabelled antigen combined to the antibody (Ag*Ab). Thus, on suitable calibration, the amount of the unlabeled species can be accurately measured in terms of the amount of radioactivity associated with the antigen-antibody complex.

A first step involves the incubation of antigens and antiserum. The incubation conditions have to ensure the stability of all reagents as antigen binds to antibody and allow equilibrium to be reached. A second step involves the separation of free and bound antigen. When equilibrium has been achieved, the antigen bound to antibody is quickly and efficiently separated from free antigen so that the radioactivity associated with either or both components can be counted. Many different separation procedures have been reported (Chard, 1978). One method is based on the ability of relatively low concentrations of polyethyleneglycol to bring about the precipitation of antibody molecules, presumably by removal of the attendant hydration shell of water molecules, without the precipitation of the smaller antigen molecules (Desbuquois and Aurbach, 1971). This method is not efficient in all cases, but is worth trying because of its simplicity and very low cost. Another method, called double antibody method, is very widely used and can achieve an efficient separation of free and bound antigen in more or less all radioimmunoassays. The basis of the most common type of this method is to use an antiserum (second antibody), raised to antibodies of the antiserum (first antibody) employed in the incubation, to precipitate the antigen-antibody complex (Midgley and Hepburn, 1980). The addition of non-immune γ-globulin of the species in which the first antiserum was raised increases the bulk of material that can interact with the second antiserum and so enables a precipitate to be formed.

Two quantitation methods were tested. A first method, described in Pantex extraction ^{125}I oestradiol kit (Catalogue n° 047), was based on the use of anti oestradiol produced in rabbits using oestradiol-6-CMO-BSA as a first antiserum and a goat produced antiserum as a second antiserum. A second method, described by Webb *et al.* (1985), was based on the use of anti oestradiol produced in rabbits (R48 22/6/83) as a first antiserum and a donkey anti rabbit

serum as a second antiserum. However, as was mentioned before, before applying the quantitation methodology, an initial step of purification of oestradiol is required to increase the specificity of the antibodies.

7.2.2. Protocols

7.2.2.1. Purification

7.2.2.1.1. Solvent extraction

1. To screw-capped and labelled glass tubes (16 x 125 mm borosilicate glass tubes with Teflon lined screw caps), 0.25 ml of plasma (containing endogenous E_2) was added.

2. To each tube, 1,000 cpm of ^3H-E_2 (recovery label, 10 µl diluted in ethanol) was added then the tube was vortexed and incubated for 15 minutes at room temperature. One Total Counts tubes was put aside for recovery estimate.

3. To each tube, 6 ml of extraction mixture (hexane : ethyl acetate, 3:2) was added.

4. The tubes were shaken for one minute then the layers were left to separate.

5. As much as possible of the upper solvent layer (5 ml) was pipetted into glass scintillation vials.

6. The solution was evaporated to dryness in the vacuum oven at 40 to 45°C overnight.

7. When dried, 0.8 ml of diluent (supplied in the kit) was added, the tubes were vortexed and placed in water bath at 37°C for 30 minutes.

8. The tubes were vortexed. 0.1 ml of reconstituted solution was pipetted into scintillation vials for recovery estimate. 0.25 ml (x 2) was pipetted into two clean glass tubes (12 x 75 mm) for assay. 0.25 ml of diluent was added into each tube to obtain a final volume of 0.5 ml (as for the calibrators).

7.2.2.1.2. Immunoaffinity purification

1. Assay buffer (Phosphate Buffered Saline) containing 0.1% gelatine (PBS-Gel) was prepared as follows : 18 g of NaCl were mixed with 200 ml of 0.5M PO_4 (pH 7.5), 300

ml of double distilled water (ddH_2O) and 2 g of Swine skin gelatine. The solution was heated up until it became transparent and made up to 2,000 ml using ddH_2O (add 1,500 ml).

2. The anti-E_2-Sepharose binding check was carried out to optimise the concentration used (500 µl with 3 ml of samples / ddH_2O).

3. The sintered glass columns were recycled by washing with three cycles of one 90% methanol and three ddH_2O (12 times in total).

4. To screw-capped glass tubes (16 x 125 mm), 0.25 ml of plasma (containing endogenous E_2) was added.

5. To each tube, 1,000 cpm of ^3H-E_2 (recovery label, 10 ml diluted in ethanol) was added. the tubes were vortexed and incubated for 30 minutes at RT (including three total and three ddH_2O tubes for recovery).

6. To each tube, 9.75 ml of ddH_2O and 0.5 ml of anti-E_2-Sepharose (optimised amount obtained by binding check) were added.

7. The tubes were then mixed end over end overnight.

8. The contents of each tube was poured on to pre-washed sintered glass columns, and the aqueous waste discarded. To rinse the antibody-sepharose residues, 7 ml ddH_2O were added to each tube, the tube was shaken and the solution was poured onto the respective columns.

9. The columns were washed with three batches of ddH_2O (21 ml) and the elutes allowed to run to waste. Residual ddH_2O in the system was then removed by applying slight positive pressure using an aquarium pump.

10. Bound hormone was eluted with 3 ml of 90% methanol into glass tubes (16 x 125 mm), and again positive pressure was applied to ensure maximum elution of the solvent into glass test tubes.

11. The glass tubes were dried down in a Buchler Vortex Evaporator at 40°C (Gallenkamp). The hormone extracts were reconstituted in 1.8 ml of PBS-Gel buffer, and then mixed for at least further 20 minutes on a Vortex evaporator.

12. The recovery was expressed a percentage of total counts by taking a 500 µl aliquot of the reconstituted material and counting on a liquid scintillation counter (Wallac 1410). Two 500 µl aliquots of the extracted and reconstituted steroid were then removed for RIA.

13. After use, the residual antibody-sepharose was re-suspended in 7 ml ddH_2O and the slurry from each column pooled in a large sintered glass funnel (porosity 3). The anti-E_2-Sepharose was recycled by washing with three alternate cycles of one 90% methanol and three ddH_2O, and was then collected from funnel and made up to original volume with ddH_2O.

7.2.2.2. Quantitation

7.2.2.2.1. Pantex ^{125}I oestradiol kit radioimmunoassay

1. The standards were prepared. 0.1 ml of stock standard was diluted in a 10 ml volumetric flask with diluent to give a concentration of 512 pg per ml. The standard curve was established as described in Table 56.

Table 56 : Preparation of standard curve.

	Concent. per ml	Concent. per tube	Volume	Diluent volume	Calibrator Serum Equivalent
1	512	256	0.1 ml of stock std	9.9 ml	1280
2	256	128	2 ml of (1)	2.0 ml	640
3	128	64	2 ml of (2)	2.0 ml	320
4	64	32	2 ml of (3)	2.0 ml	160
5	32	16	2 ml of (4)	2.0 ml	80
6	16	8	2 ml of (5)	2.0 ml	40
7	8	4	2 ml of (6)	2.0 ml	20
8	4	2	2 ml of (7)	2.0 ml	10

2. 0.5 ml of the samples (0.25 ml reconstituted extract samples or control + 0.25 ml diluent) and 0.5 ml of diluted calibrators were pipetted into glass tubes (12 x 75 mm). 0.5 ml diluent were used for NSB and 0 calibrator tubes. Two quality controls were included.

3. 0.1 ml of ^{125}I-oestradiol tracer were pipetted into each tube (0.9 µCi per tube).

4. 0.1 ml of **first antiserum** (Rabbit anti E_2) was added to all tubes except NSB and total counts (TC) tubes. 0.1 ml of NSB buffer were added to NSB tubes. The tubes were vortexed and incubated in a water bath at 37°C for 2 hours.

5. 0.5 ml of **second antiserum** (Goat anti-Rabbit) was added to all tubes except TC tubes. The tubes were vortexed and incubated at room temperature for 30 minutes.

6. The tubes were centrifuged at 3500 rpm for 10 minutes. The supernatant was aspirated and discarded without disturbance of the precipitates. The tubes were drained for approximately 2 minutes. The liquid on tube rim was blotted with tissue.

7. The tubes were counted in γ-counter (1277 automatic γ-counter, Wallac).

7.2.2.2.2. Webb et al. (1985) radioimmunoassay

1. 2 batches of standards were prepared each in triplicate from the stock solution (100 pg per ml) to obtain 12 points : 0.5, 0.75, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, 16.0, 24.0 and 48.0 pg per tube and made to 500 µl per tube with Phos-Gel.

2. The samples were prepared in duplicate, made up to 500 µl with Phos-Gel.

3. 0.1 ml ^{125}I-oestradiol tracer were pipetted into each tube (0.9 µCi per tube).

4. 0.2 ml of first antibody (R48 22/6/83 1:100; diluted to 1:40,000, 150 µl in 60 ml of Phos-Gel) was added to all tubes except TC and blank.

5. The tubes were vortexed, incubated for at least two hours at RT.

6. 0.1 ml of Normal Rabbit Serum (1:400) was added to increase the precipitate to all tubes except TC.

7. 0.1 ml of the second antibody raised in Donkey to Rabbit IgG (1:40) was added to all tubes except TC and 1:10 EDTA (0.1M in PBS) were also added to help precipitation (chelative agent containing Ca, Mg) in the same tubes.

8. The tubes were vortexed and left in a cold room (4°C) overnight.

9. The next day, 1 ml cold Phos-Gel was added to increase the volume.

10. The tubes were spinned at 3,700 rpm for 25 minutes (PR-7000, International Equipment

Company).

11. The supernatant was poured and the pellet was left to dry for a few seconds and counted in γ-counter (1277 automatic gamma counter, Wallac).

7.2.2.2.3. Calculation

1. The $\%B/B_o$ of calibrators (binding relative to 0 calibrator) was calculated. These binding ratios were calculated from net CPM values. Net CPM = (mean CPM) - (NSB mean CPM).

$$\%B/B_o = \frac{\text{net CPM of calibrator}}{\text{net CPM of 0 calibrator}} \times 100$$

2. Points relating calculated $\%B/B_o$ values and calibrator concentrations were plotted on logit-log graph paper (Rodbard *et al.*, 1969). The best line was drawn through these points.

3. The $\%B/B_o$ values of samples were calculated.

$$\%B/B_o = \frac{\text{net CPM of sample}}{\text{net CPM of 0 calibrator}} \times 100$$

4. The $\%B/B_o$ values were interpolated on line. The points were projected on the X-axis and the intersections marked the oestradiol concentrations.

5. The results (expressed in pg per tube) were divided by the volume of reconstituted sample used for RIA and multiplied by the volume of diluent or PBS-Gel buffer used to reconstitute the hormone extracts. The results were expressed in pg of reconstituted volume.

6. Recovery was calculated by dividing recovery counts by the volume of reconstituted sample used for recovery estimation and multiplied by the volume of diluent or PBS-Gel buffer used to reconstitute the hormone extracts. The result was then divided by the mean total recovery counts added.

7. The results obtained in (5.) were then divided by those obtained in (6.) to obtain the amount of 17β-oestradiol in pg in the original volume of plasma (i.e. 0.25 ml).

8. The results obtained were divided by 0.25 ml to obtain the concentration of 17β-oestradiol in pg per ml plasma.

7.3. Experiment 6

The objective of this experiment was to compare the results of the methods previously described to determine which of the two extraction methods and which of the two radioimmunoassay methods gave the more adequate results.

7.3.1. Materials and methods

To appreciate the adequacy of the results, the 17β-oestradiol concentration was assayed in four samples of plasma, each samples being spiked with different amounts of 17-β oestradiol. The oestradiol standard was obtained in the Pantex kit (oestradiol stock calibrator) and contained 51,200 pg of oestradiol per ml. This solution was diluted with diluent (from the kit) to obtain different oestradiol concentrations : 75, 150, 300, 450 and 600 pg per ml. Each of these solutions was then added to the plasma samples as follows :

Tube a : 0.9 ml of plasma + 0.1 ml of diluent
Tube b : 0.9 ml of plasma + 0.1 ml of oestradiol 75 pg per ml
Tube c : 0.9 ml of plasma + 0.1 ml of oestradiol 150 pg per ml
Tube d : 0.9 ml of plasma + 0.1 ml of oestradiol 300 pg per ml
Tube e : 0.9 ml of plasma + 0.1 ml of oestradiol 450 pg per ml
Tube f : 0.9 ml of plasma + 0.1 ml of oestradiol 600 pg per ml

Four plasma samples, three from a 14-week old hen and one from a 2-week old broiler, were spiked. Each spiked sample was then divided into four samples of 0.2 ml. Two of them were analysed by the kit method (one sample was extracted with solvent, the other by affinity chromatography), the two other are analysed with Webb *et al.* (1985) technique (one sample was extracted with solvent, the other by affinity chromatography).

7.3.2. Results

As was described previously, the RIA methods were based on the use of a standard solution of oestradiol (stock calibrator) to establish a regression line. The binding ratios, $\%B/B_o$, were calculated for each oestradiol calibrator concentration (as described in chapter 7.2.2.2.3.) and

were plotted on a logit-log paper with in ordinate the percent bound and in abscissa the concentration of oestradiol in pg per ml. The graphs obtained with the kit and Webb *et al.* (1985) methods are given in Figure 23 and Figure 24.

Figure 23 : Logit Log plot of oestradiol calibrators obtained with the kit method.

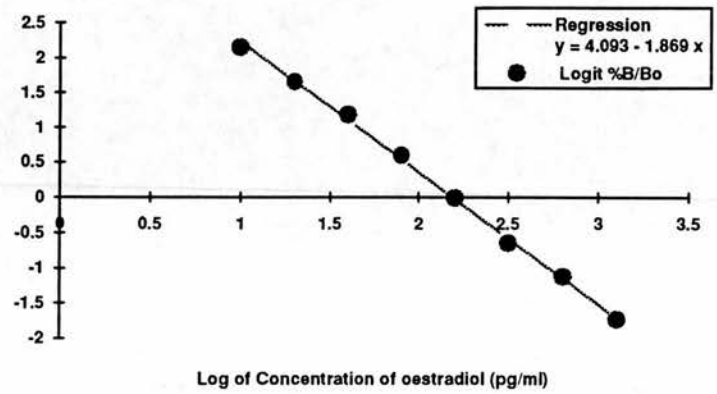
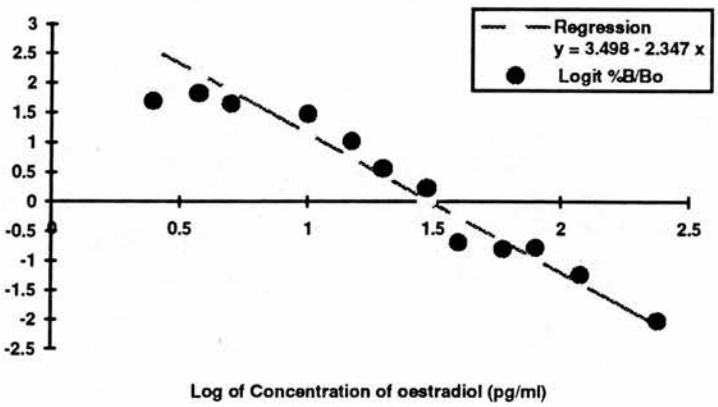


Figure 24 : Logit Log plot of oestradiol calibrators obtained with Webb *et al.* (1985) method.



The interpolation of the $\%B/B_o$ ratios of the spiked samples on these lines allowed the determination of their oestradiol concentrations (Table 57).

Table 57 : Oestradiol concentration (in pg per ml) in spiked samples.

Tube	Kit method		Webb et al. method	
	Solvent extraction	Affinity chromatography extraction	Solvent extraction	Affinity chromatography extraction
1a	33.6	23.4	211.1	83.8
1b	36.3	46.2	224.7	86.5
1c	41.9	99.0	351.9	92.8
1d	46.7	46.7	399.8	103.6
1e	53.1	24.4	364.7	107.0
1f	70.9	96.3	424.5	99.1
2a	45.9	69.0	311.8	53.6
2b	58.4	91.7	335.8	77.7
2c	64.8	65.6	213.6	83.5
2d	65.1	59.1	218.1	86.7
2e	77.1	74.1	243.7	98.2
2f	81.1	54.9	227.9	113.4
3a	56.7	89.2	200.3	74.2
3b	61.3	67.0	301.8	-
3c	64.0	87.4	369.3	82.4
3d	68.6	70.5	423.8	94.5
3e	-	33.9	331.0	107.5
3f	-	94.3	436.1	128.1
4a	12.1	-	172.9	33.6
4b	17.0	-	145.3	35.1
4c	23.2	-	152.0	40.9
4d	33.4	43.4	158.2	47.5
4e	31.4	37.1	214.9	50.5
4f	50.6	120.3	359.6	62.3

7.3.3. Discussion

Depending on the method of extraction and quantitation used, the determined concentrations of oestradiol were very different. As an average concentration of oestradiol of 80 to 100 pg per ml of plasma was reported in 14-week aged hens (Senior, 1974), the plasma samples extracted by solvent and analysed by Webb *et al.* (1985) quantitation technique did not seem to give appropriate results. These results showed much higher oestradiol concentrations for Tubes 1a, 2a and 3a (with respectively 211.1, 311.8 and 200.3 pg per ml) than those reported by Senior. In contrast, the samples analysed with the kit method showed, whatever the extraction method, lower results than those reported by Senior with concentrations of 33.6, 45.9 and 56.7 pg per ml for the samples extracted with solvent and of 23.4, 69.0 and 84.0 pg per ml for those extracted with affinity chromatography. Although the last samples, extracted with affinity chromatography, seemed to have produced more similar results to those of Senior, the low

consistency of the results, which should have increased from Tubes a to f, made this technique impossible to use.

The only technique, which seemed to provide appropriate results, was therefore the one where the samples were extracted by affinity chromatography and then analysed according to Webb *et al.* (1985) RIA method. The results were similar to those found by Senior, with concentrations of 86.5, 53.6 and 74.2 pg per ml for the 1a, 2a and 3a Tubes and showed a more consistent increase between Tubes a to f. The technique that was used to determine oestradiol concentration in the next experiment was therefore the one in which the plasma were extracted with affinity chromatography and then analysed with Webb *et al.* (1985) technique.

7.4. Experiment 7

The objective of this experiment was to investigate the effect of a dietary vitamin E supplementation on the plasma concentration of 17β-oestradiol in laying hens exposed to thermoneutral conditions or heat stress conditions.

7.4.1. Materials and methods

This experiment was carried out on Isa Brown birds aged between 23 and 30 weeks. These birds were fed either a control diet with a vitamin E level of 10 mg per kg of feed (commercial diet) or a vitamin E supplemented diet containing 250 mg of vitamin E per kg diet from 23 weeks of age. The temperature was fixed to 22°C from the 23th to the 26th weeks of age of the birds (28 days) and increased to 32°C between the 27th to the 30th weeks of age of the birds (for another 28 days). The relative humidity was constant and fixed to 75%. Blood samples were taken from twenty control and ten supplemented birds on two occasions during the thermoneutral period (at the ends of the 25th and 26th weeks of age of the birds) and on two occasions on the same birds during the heat stress period (at the ends of the 29th and 30th weeks of age of the birds).

7.4.2. Results

The results are given in Table 58.

Table 58 : **Influence of heat stress or vitamin E on the concentration of 17β-oestradiol in the plasma (in pg per ml).**

	Plasma 17β-oestradiol concentration (pg per ml)					
	Thermoneutral period			Heat stress period		
	Week 25	Week 26	Mean	Week 29	Week 30	Mean
Control birds	254 ± 59 (n=11)	252 ± 112 (n=8)	**253 ± 86 (n=19)**	259 ± 98 (n=11)	240 ± 83 (n=7)	**252 ± 93 (n=18)**
Vit.E treated birds	225 ± 88 (n=8)	238 ± 57 (n=5)	**230 ± 78 (n=13)**	189 ± 66 (n=3)	205 ± 71 (n=4)	**198 ± 69 (n=7)**

7.4.3. Discussion

The results of this experiment demonstrated that neither heat stress nor vitamin E affected the concentration of 17β-oestradiol in the plasma of laying hens. These results are at variance with those of Mahmoud *et al.* (1995) who found a reduction of circulating 17β-oestradiol concentration in hens exposed to heat stress (35°C, 50% R.H.). However, as these authors did not mention the duration of the stress period, it is possible that the absence of response in experiment 7 is due to the long duration of the heat stress period. Indeed the samples analysed were taken three and four weeks after the beginning of the hot period and it is possible that, after such a duration, the birds had recovered their normal 17β-oestradiol concentration in the plasma. Studies carried out on hens stressed for a long period (13 weeks at 31°C) showed that the circulating concentration of 17β-oestradiol was increased in these birds compared to this of birds maintained at 21°C (Erb *et al.*, 1978).

What may also explain the lack of response, is the fact that, for practical reasons, the samples were collected from the birds at a fixed time in the day (between 8 and 10 a.m.) without regards to the individual times of oviposition. However, as the concentration 17β-oestradiol in the circulation varies according to a cyclic pattern related to oviposition, a better appreciation of the hormone concentration would probably have been achieved if the samples would have been collected at fixed times after oviposition.

7.5. Conclusions

Experiment 6 suggested that the use of the methods of purification by affinity chromatography extraction and analysis by radioimmunoassay as described by Webb *et al.* (1985) were adequate for the determination of the concentration of 17β-oestradiol in the plasma of layers. Experiment 7 showed that neither a period of 4 weeks of heat stress nor the supply of vitamin E had any significant effect on the circulating concentration of 17β-oestradiol at the end of the period of heat stress. It can be argued that the individual oviposition time should have been taken into account in the schedule of sampling but there is no reason to believe that it would have change the average concentrations of 17β-oestradiol observed in the groups. Sampling at a specific time in relation to oviposition might have reduced the standard deviation of each mean, but there is a very low probability that it would have demonstrated a difference between the thermoneutral and heat-stressed birds. It is also not probable that it would have reversed the results and demonstrated a higher 17β-oestradiol concentration in the vitamin E-supplemented group compared to the non-supplemented group.

As a result, and provided that the methodology used to measure the concentration of oestrogen was appropriate, the application of a four-week heat stress exposure on laying hens, which resulted in a noticeable reduction in egg production (Experiment 3), did not appear to affect the circulating 17β-oestradiol concentration in the birds. This result was at variance with the observations of Mahmoud *et al.* (1995) who demonstrated a decrease of the circulating concentration of 17β-oestradiol in heat stressed laying hens, but confirmed those of Erb *et al.* (1978) who subjected the birds to a long period of stress. In conclusion, the involvement of 17β-oestradiol in the response of egg production to heat stress and vitamin E supplementation can probably be excluded.

Another possible explanation for the reduction in the concentration of vitellogenin in the plasma of laying hens exposed to a 4-week heat stress could be that the synthesis of vitellogenin is depressed in the liver during heat stress and that supplementation with vitamin E alleviates, at least in part, this effect. This hypothesis was studied in the experiments described in the next chapter.

Chapter 8

Effect of chronic heat stress and vitamin E on the levels of vitellogenin mRNA and protein in laying hens

The work described in the previous chapters demonstrated that dietary vitamin E supplementation alleviated the detrimental effect of heat stress on egg production in laying hens. To understand the mechanisms by which vitamin E influences egg production, the main egg yolk precursor protein, vitellogenin, was studied. Vitellogenin is synthesised in the liver as a high molecular weight precursor in response to oestradiol. It is secreted into the serum, and is then taken up and processed by the ovary (Bergink and Wallace, 1974).

Utomo *et al.* (1994) showed that during heat stress, the vitellogenin concentration in the plasma was reduced by 27% in control birds and 10% in supplemented birds (500 mg vitamin E per kg). The data presented thus far in this thesis show that heat stress or vitamin E supplementation had no effect on the uptake of vitellogenin in the oocytes (Chapter 6) or on the concentration of circulating 17β-oestradiol (Chapter 7).

The next experiment was therefore carried out to determine whether heat stress and vitamin E have an effect on the steady state level of the vitellogenin message. The steady state level of vitellogenin messenger RNA in a hepatocyte at a given time, which determines the amount of vitellogenin protein that can be synthesised, depends on the relative contribution of two processes. These are the transcriptional activity of the vitellogenin gene and the rate of clearance of vitellogenin mRNA. Indeed, a reduced amount of vitellogenin mRNA in an hepatocyte could result either from lower transcriptional activity of the gene encoding for vitellogenin, or from a greater rate of destruction of the vitellogenin mRNA, or from both.

8.1. Experiment 8

The objective of experiment 8 was to investigate the effect of heat stress and vitamin E on the steady state of vitellogenin mRNA. The amount of vitellogenin mRNA was therefore examined in the liver of control and heat-stressed birds and in the liver of vitamin E supplemented and non-supplemented birds. Additional determination of the amount of vitellogenin protein in the liver and plasma was also carried out to establish to what extent they relate to the amount of mRNA in the liver.

8.1.1. Materials and methods

8.1.1.1. Selection of the birds

Twenty-four Isa Brown birds aged 21 weeks were transferred to a climate-controlled room maintained at 22°C, 60% R.H. (thermoneutral conditions). The birds were fed a standard diet (10 mg of vitamin E per kg) for one week (adaptation period). Egg number and weight and body weight of the birds were recorded as described in Chapter 3. On the eighth day, the birds were distributed into twelve pairs according to their egg production and body weight. Birds showing similar egg production and body weight were paired, one bird receiving the same diet as before (10 mg of vitamin E per kg), the other receiving a vitamin E supplemented diet (300 mg of vitamin E per kg). The birds were then distributed into two groups (Group A and Group B) of twelve birds each, one group receiving a control diet, the other receiving a vitamin E supplemented diet. The characteristics of these groups are given in Table 59.

Table 59 : Characteristics of the birds before the experiment (mean ± S.D.)

	Group A (n=12)	**Group B (n=12)**
Egg production (No eggs per hen per day x 100)	84.72 ± 12.65	87.50 ± 13.82
Mean egg weight (g per egg)	51.52 ± 6.67	50.80 ± 3.98
Total egg mass (g per hen per day)	43.37 ± 6.45	44.59 ± 8.68
Body weight (g per hen)	1780 ± 97	1772 ± 114

The birds were fed these diets for 14 days and the temperature and humidity were maintained at 22°C, 60% R.H. Then five pairs of 10 birds were blood sampled, and slaughtered. Samples of liver were collected as soon as the birds were killed to minimise degradation of RNA by endogenous ribonucleases and total RNA was prepared as described below. The remaining birds were exposed to a temperature of 32°C for a week and 35°C for a further week. The relative humidity was maintained at a higher level of 70% during the two weeks. At the end of this heat stress period, blood was sampled from five pairs of birds and their livers were dissected for total RNA preparation.

8.1.1.2. Method for the determination of vitellogenin gene transcription

The level of vitellogenin mRNA was compared between control and heat stressed birds and between vitamin E treated and untreated birds using the technique of Northern hybridisation analysis. Since the site of vitellogenesis is the liver, total RNA was prepared from the livers of the above birds.

8.1.1.2.1. Isolation of total RNA from liver samples

Total RNA was isolated by single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture as described by Chomczynski and Sacchi (1987). Guanidinium thiocyanate is one of the most effective protein denaturants known. The use of guanidinium to lyse cells was originally developed to allow purification of RNA from cells high in endogenous ribonucleases (Cox, 1968 ; Ullrich *et al.*, 1977 ; Chirgwin *et al.*, 1979). This method provides a pure preparation of undegraded RNA in high yield and can be completed within 4 h. It is based on the property of RNA to remain water soluble in a solution containing 4 M guanidinium thiocyanate at pH 4 in the presence of a phenol/chloroform organic phase. Under these acidic conditions, most proteins and small fragments of DNA (50 bases to 10 kbases) are in the organic phase while larger fragments of DNA and some proteins remain in the interphase. The fragmentation of DNA during homogenisation helps to remove DNA from the water phase. Since its introduction (Chomczynski and Sacchi, 1987), the single-step method has become a widely used method for isolating RNA from a large number of samples.

The experimental protocol described below was applied. All steps were carried out at room temperature unless otherwise stated. The reagents used in this protocol were prepared as

described in section 8.1.2.5.

1. **As soon as** the bird was killed, the liver was excised and a 0.5 g (approximate) sample was transferred into pre-chilled denaturing solution (5 ml denaturing solution for 0.5 mg of tissue). The rest of the liver was wrapped in a sheet of pre-baked (to inactivate ribonucleases) aluminium foil and quickly frozen in liquid nitrogen before storage at -80°C.
2. The sample of liver was homogenised in denaturing solution with a polytron homogeniser. The homogenate was transferred to a 14-ml polypropylene tube.
3. 0.5 ml of 2M sodium acetate (pH 4) was added and the contents of the tube were thoroughly mixed by vortexing. 5 ml water-saturated phenol was added with vortexing followed by 1 ml of chloroform. The solution was thoroughly mixed by vortexing for one minute and the suspension was then incubated for 15 minutes on ice.
4. The suspension was centrifuged for 20 min at 10,000 x g, 4°C. The upper aqueous phase was transferred to a fresh tube. *The volume of the aqueous phase was approx. 5 ml, equal to the initial volume of denaturing solution.*
5. The RNA was precipitated by adding 5 ml of isopropanol. The samples were placed for 30 min (or overnight) at -20°C. They were then centrifuged for 10 minutes at 10,000 x g, 4°C, and the supernatant was discarded. Liver tissues are often contaminated by glycogen (Puissant and Houdebine, 1990). Therefore after this precipitation, glycogen was washed out from the RNA pellet by vortexing in 1 ml of 4M LiCl. The insoluble RNA was pelleted by centrifugation for 10 minutes at 5,000 x g.
6. The pellet was dissolved in 0.5 ml of denaturing solution and transferred into a 1.5 ml microcentrifuge tube.
7. The RNA was precipitated with 1 ml of 100% ethanol for 30 min (or overnight) at -20°C. The solution was centrifuged for 10 minutes at 10,000 x g, 4°C, and the supernatant was discarded.
8. The RNA pellet was resuspended in 70% ethanol by vortexing, and incubated for 10 to 15 minutes at room temperature to dissolve residual amounts of guanidinium contaminating the pellet.
9. The solution was centrifuged for 5 minutes at 10,000 x g and the supernatant was removed by aspiration.
10. The RNA pellet was dissolved in a suitable volume of DEPC-treated water. The samples

were stored frozen at -70°C. With this method, approximately 4 mg of total RNA was isolated per 0.5 g of tissue.

8.1.1.2.2. Quantification of total RNA in the samples

To quantify the RNA in each sample, spectrophotometric measurement of the amount of ultraviolet irradiation absorbed by the bases was performed. Readings were taken at 260 and 280 nm. The reading at 260 nm allows calculation of the concentration of RNA in the sample. DNA also absorbs at 260 nm. However, the method used to isolate total RNA consistently gives RNA preparations that are free of genomic DNA contamination. It was therefore assumed that the absorbance due to DNA, if any, in the preparation was minimal. An optical density (O.D.) of 1 corresponds to 40 µg per ml of RNA. The ratio between the readings at 260 and 280 nm ($O.D._{260}$ / $O.D._{280}$) provided an estimate of the purity of the RNA preparation. Pure preparations of RNA have $O.D._{260}$ / $O.D._{280}$ values of about 2.0.

8.1.1.2.3. Analysis of RNA by electrophoresis in formaldehyde gels

1. Preparation of the gel (for 400 ml) :

- add 3.6 g agarose (for 0.9% gel) to 290 ml DEPC water. The agarose used was Seakem from FMC Bioproducts (Cat N° 50004).
- the agarose was dissolved by microwaving and then cooled to 60°C.
- 40 ml 10 x MAE and 70 ml formaldehyde (final concentration 2.15M) (BDH AnalaR grade) were added. The final volume was adjusted to 400 ml with DEPC water.

It was critical to set aside a gel tank for RNA work to avoid contamination with ribonucleases. Before use, the tank was rinsed with DEPC water.

2. Preparation of the samples :

- to 10 µg of each total RNA sample, 16 µl formaldehyde mix was added.
- the sample was heated at 65°C for 10 minutes.
- 3.5 µl dye and 0.5 µl ethidium bromide solution (10 mg per ml) were added.

3. The samples were electrophoresed at 30 V overnight with 1 x MAE as running buffer.

8.1.1.2.4. Transfer of RNA onto nylon membranes

1. Following electrophoresis, the gel was shaken sequentially in a tray of distilled water for one hour, 50 mM sodium hydroxide (freshly prepared) for 45 minutes and 0.5 M sodium phosphate buffer pH 7.2 for 30 minutes.
2. Nylon membrane (Hybond N^+, Amersham) was pre-wet sequentially with distilled water and then 0.5 M sodium phosphate buffer.
3. The RNA samples were allowed to transfer overnight by capillary action in a "dry" set-up. The gel was inverted on a glass plate and overlaid with Hybond N^+ (making sure that all air bubbles were smoothed out), 3 sheets Whatman paper (wet in 0.5 M sodium phosphate buffer), another 3 sheets of dry Whatman paper, a stack of paper towels and a second glass plate with suitable weight on top.
4. The next morning, the gel sandwich was disassembled and orientation markings were made on filter.
5. The filter was rinsed briefly in 50 mM sodium phosphate buffer and subjected to UV crosslinking (one autocrosslink cycle in a Stratalinker).
6. The membrane was then hybridised with the appropriate radiolabelled cDNA probe.

8.1.1.2.5. Preparation of the vitellogenin and 18S rRNA cDNA probes. Hybridisation

The first step in this procedure was to obtain plasmids carrying the cDNA fragments of interest. The plasmid containing the chicken vitellogenin cDNA fragment was kindly provided by Dr G. Ab of the Dept of Biochemistry, University of Groningen in the Netherlands (van het Schip *et al.*, 1987). The plasmid containing the chicken 18S ribosomal RNA cDNA fragment was kindly provided by Dr. M. Clinton of the Roslin Institute.

As only a small amount of each plasmid was provided, they had to be amplified. Each plasmid was introduced by electroporation into a bacterial host E. coli strain DH10B provided by K. Lee (from the Centre for Genome Research, University of Edinburgh). The bacteria were grown in standard LB culture medium in the presence of a selective agent: ampicillin (antibiotic) to ensure that all the cells that grow contain the plasmid. Once enough bacterial cells were obtained (approximately 10^9 cells per ml), they were harvested by centrifugation at 6,000 g for 15 minutes at 4°C. All traces of supernatant were removed by inverting the open

centrifuge tube until all medium had been drained. The plasmid DNA was then prepared and purified using a commercially available kit (QIAGEN) according to the manufacturer's instructions. The protocol used was for preparation of approx. 0.1 mg of plasmid DNA and the main steps were as follows :

1. The bacterial pellet was resuspended in 4 ml of a Buffer P1 containing RNase A. The pellet was resuspended completely, leaving no cell clumps.
2. 4 ml of NaOH/SDS (Buffer P2) was added to lyse the bacterial cells. The solution was mixed gently by inverting the tube 4-6 times and incubated at room temperature for no more than 5 minutes. SDS solubilises the phospholipid and protein components of the cell membrane, leading to lysis and release of the cell contents. NaOH denatures the chromosomal and plasmid DNAs, as well as proteins. The optimised lysis time allows maximum release of plasmid DNA without release of chromosomal DNA, whilst minimising the exposure of the plasmid to denaturing conditions.
3. 4 ml of pre-chilled acidic potassium acetate (Buffer P3) was added to neutralise the lysate. To avoid localised potassium dodecyl sulphate precipitation, the solution was mixed immediately by inverting the tube 5 to 6 times and incubated on ice for 15 minutes. The high salt concentration causes SDS to precipitate, and the denatured proteins, chromosomal DNA, and cellular debris become trapped in salt/detergent complexes. Plasmid DNA, being smaller and covalently closed, renatures correctly and remains in solution. Since any SDS remaining in the lysate will inhibit binding of DNA to the QIAGEN resin, the solution must be gently but thoroughly mixed to ensure complete precipitation of the detergent.
4. The sample was mixed again and centrifuged at 20,000 x g for 15 minutes at 4°C in a non-glass tube. After centrifugation, the clear supernatant was removed promptly.
5. The supernatant was centrifuged again at 20,000 g for a further 15 minutes at 4°C. This step was carried out to avoid applying suspended or particulate material to the QIAGEN-tip. Suspended material (causing the sample to appear turbid) can clog the QIAGEN-tip and reduce or eliminate gravity flow.
6. The QIAGEN-tip was equilibrated by applying 4 ml of Buffer QBT, and the column was allowed to empty by gravity flow.
7. The supernatant was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The salt and pH conditions in the lysate and the superior selectivity of the QIAGEN

resin ensure that only plasmid DNA binds, while degraded RNA, cellular proteins, and metabolites are not retained, and appear in the flow-through fraction.

8. The QIAGEN-tip was then washed twice with 10 ml of buffer containing 1 M NaCl (Buffer QC) which completely removes any remaining contaminants, such as traces of RNA and protein (e.g. RNase A), without affecting the binding of the plasmid. This buffer also disrupts non-specific interactions, and allows removal of nucleic acid-binding proteins without the use of phenol. The low concentration of alcohol in the wash buffer eliminates nonspecific hydrophobic interactions, further enhancing the purity of the bound DNA.

9. The DNA was eluted from the QIAGEN-tip with 5 ml of a buffer containing 1.25 M NaCl at pH 8.5 (Buffer QF). The eluate was collected in a tube resistant to ethanol which is used in subsequent steps.

10. The DNA was precipitated with 3.5 ml of isopropanol and mixed. Precipitation of DNA was carried out with all solutions at room temperature in order to minimise salt precipitation. The solution was then immediately centrifuged at 15,000 g for 30 minutes (at 4°C to prevent overheating of the sample) and the supernatant was carefully removed.

11. The DNA pellet was then briefly washed with 2 ml of 70% ethanol, and recentrifuged. The 70% ethanol serves to remove precipitated salt, as well as to replace isopropanol with the more volatile ethanol, making the DNA easier to redissolve. After careful and complete removal of ethanol, the pellet was air-dried briefly (approximately 5 minutes) before redissolving in an appropriate volume of TE buffer (pH 8.0) and was ready for use.

Once the plasmid DNA were isolated, the inserts of interest were released by digestion with restriction enzymes. Hind III & Sma I were used to digest a fragment of 470 base pairs for the vitellogenin cDNA and EcoR I was used to digest a fragment of 256 base pairs for the 18S rRNA cDNA. The restriction digests were fractionated by electrophoresis on an agarose gel (cast in 1 x TAE) containing ethidium bromide (0.5 μg per ml). The piece of gel containing the band of interest was existed with a clean scalpel under long wavelength ultraviolet illumination and DNA recovered using the QIAEX II extraction kit (Qiagen) according to the manufacturer's instructions. The latter relies on the ability of a high concentration chaotropic salt solution to disrupt hydrogen bonding between sugars in the agarose polymer, thus solubilising the gel slice. The liberated DNA was bound to a silica resin, subjected to a series of washing steps which eliminate non-nucleic acid impurities and finally eluted in TE or water.

DNA fragments were labelled to high specific activity (usually at least 1 x 10^9 dpm per μg) by the random priming method (Feinberg and Vogelstein, 1983). Typically 25 to 50 ng of the template in a volume of 11 μl was denaturated by boiling for 5 minutes and then chilled on ice for 5 minutes. 5 μl (50 μCi) of [α^{32}P]dCTP (3,000 Ci/mmol) and 4 μl of High Prime (Boehringer) were added and the whole mixture incubated at 37°C for at least 30 minutes. High Prime is an optimised mixture of dATP, dGTP, dTTP, random hexanucleotides and Klenow polymerase. Unincorporated label was removed by gel filtration through a Sephadex G-50 column.

The membrane was then hybridised overnight with the radiolabelled cDNA probe in the presence of 7% SDS, 0.25 M sodium phosphate buffer and 1mM EDTA. The next morning, the membrane was washed to a final stringency of 0.1 x SSC, 0.1% SDS at 68°C.

8.1.1.2.6. Reagents and solutions

DEPC-treated water

Diethyl pyrocarbonate (DEPC) was added to deionised water to a final concentration of 0.1%. The solution was incubated overnight at room temperature in a fume hood and then autoclaved for 20 minutes to inactivate the DEPC. Care was taken when handling DEPC as it is a suspected carcinogen.

Denaturing solution

A stock solution, containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.1 M 2-ME and 0.5% N-lauroylsarcosine (Sarkosyl) was prepared by dissolving 250 g guanidinium thiocyanate in 293 ml H_2O, 17.6 ml of 0.75 M sodium citrate, pH 7, and 26.4 ml of 10% Sarkosyl at 60° to 65°C with stirring. This solution could be stored up to 3 months at room temperature. The working solution was prepared by adding 0.35 ml of 2-ME per 50 ml of stock solution and could be stored for up to one month at room temperature.

2M sodium acetate

16.42 g of sodium acetate (anhydrous) was added to 40 ml H_2O and 35 ml of glacial acetic acid. The pH of the solution was adjusted to 4 with glacial acetic acid and the final volume was adjusted to 100 ml with H_2O. The solution was 2 M with respect to the sodium ions.

10 x MAE

This solution was prepared by mixing together 400 ml of 1 M 3-(N-morpholino) propanesulfonic acid (209.3 g per litre), 33 ml of 3 M sodium acetate pH 5.2 and 20 ml of 0.5 M EDTA (pH 8.0). The volume was adjusted to 1 litre with distilled water and sterile filter.

Formaldehyde mix

This solution was prepared by mixing together 40 µl of 10 x MAE, 40 µl of DEPC water, 0.14 ml of formaldehyde and 0.4 ml formamide (ultrapure).

Dye

Composition : 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol.

Sodium phosphate buffer

This solution was prepared by mixing together 134 g of $NaHPO_4$ 7 H_2O to 4 ml of 85% H_3PO_4 (orthophosphoric acid, BDH, analaR) and the volume was made up to 1 litre with distilled water.

20 x SSC

This solution was prepared by mixing together 175.3 g of NaCl, 88.2 g of trisodium citrate and the volume was adjusted to 1 litre with distilled water.

50 x TAE

This solution was prepared by mixing together 242 g of Trisbase, 57.1 ml of glacial acetic acid, 100 ml of EDTA 0.5 M (pH 8.0) and the volume was adjusted to 1 litre with distilled water

TE

This solution was prepared by mixing together 10 mM of TrisCl (pH 8.0) and 1 mM of EDTA (pH 8.0).

8.1.1.3. Method for the determination of yolk precursors concentration in the liver

In addition to the samples collected for RNA preparation, a sample of approximately 3.5 g of

liver was collected from each bird and placed in 10 ml of 0.9% saline. The sample was homogenised using a polytron homogeniser and centrifuged at 4,000 g for 10 minutes. The supernatant was collected, diluted 4-fold with 0.9% saline and assayed for vitellogenin-bound zinc and triglyceride concentration as described in sections 3.2.3 and 3.2.4. respectively.

8.1.2. Results

8.1.2.1. Overall performance

8.1.2.1.1. Performance of the birds during the thermoneutral period

Data on egg production parameters, feed intake and body weight of the birds during the two-week thermoneutral period are presented in Table 60. These results confirmed that, during thermoneutral period, the mean characteristics of both groups were very similar.

Table 60 : Characteristics of the birds during the thermoneutral period (mean ± S.D.)

	Control group (Group A) (n=12)	**Vit.E-suppl. group (Group B) (n=12)**
Egg production (No eggs per hen per day x 100)	94.64 ± 6.60	90.47 ± 13.78
Mean egg weight (g per egg)	52.77 ± 2.46	53.80 ± 2.92
Total egg mass (g per hen per day)	49.96 ± 4.37	48.96 ± 8.93
Feed intake (g per hen per day)	111.2 ± 10.7	114.2 ± 11.1
Body weight (g per hen)	1791 ± 105	1780 ± 100

8.1.2.1.2. Performance of the birds during the heat stress period

The production parameters, feed intake and body weight of the birds are presented in Table 61. During the heat stress period, total egg mass, feed intake and body weight declined ($P<0.01$) for the birds receiving the control diet (Group A). Total egg mass dropped from 49.96 to 37.59 g per day per hen during the stress for the birds fed the control diet ($p<0.01$). However,

for the birds receiving the vitamin E supplemented diet (Group B), total egg mass was not affected by heat stress and was similar to what was observed before the stress, i.e. 48.96 g per day per hen before the stress and 46.80 g per day per hen during the stress. This result was consistent with previous observations (Chapter 4) showing that vitamin E alleviates the effect of heat stress on egg production.

Table 61 : Characteristics of the birds during the heat stress period (mean ± S.D.)

	Control group (Group A) (n=7)	Vit.E-suppl. group (Group B) (n=7)
Egg production (No eggs per hen per day x 100)	72.44 ± 22.07	89.79 ± 5.20
Mean egg weight (g per egg)	51.97 ± 3.18	52.20 ± 2.19
Total egg mass (g per hen per day)	37.59 ± 11.89	46.80 ± 1.87
Feed intake (g per hen per day)	58.9 ± 10.8	68.3 ± 5.2
Body weight (g per hen)	1516 ± 136	1543 ± 32

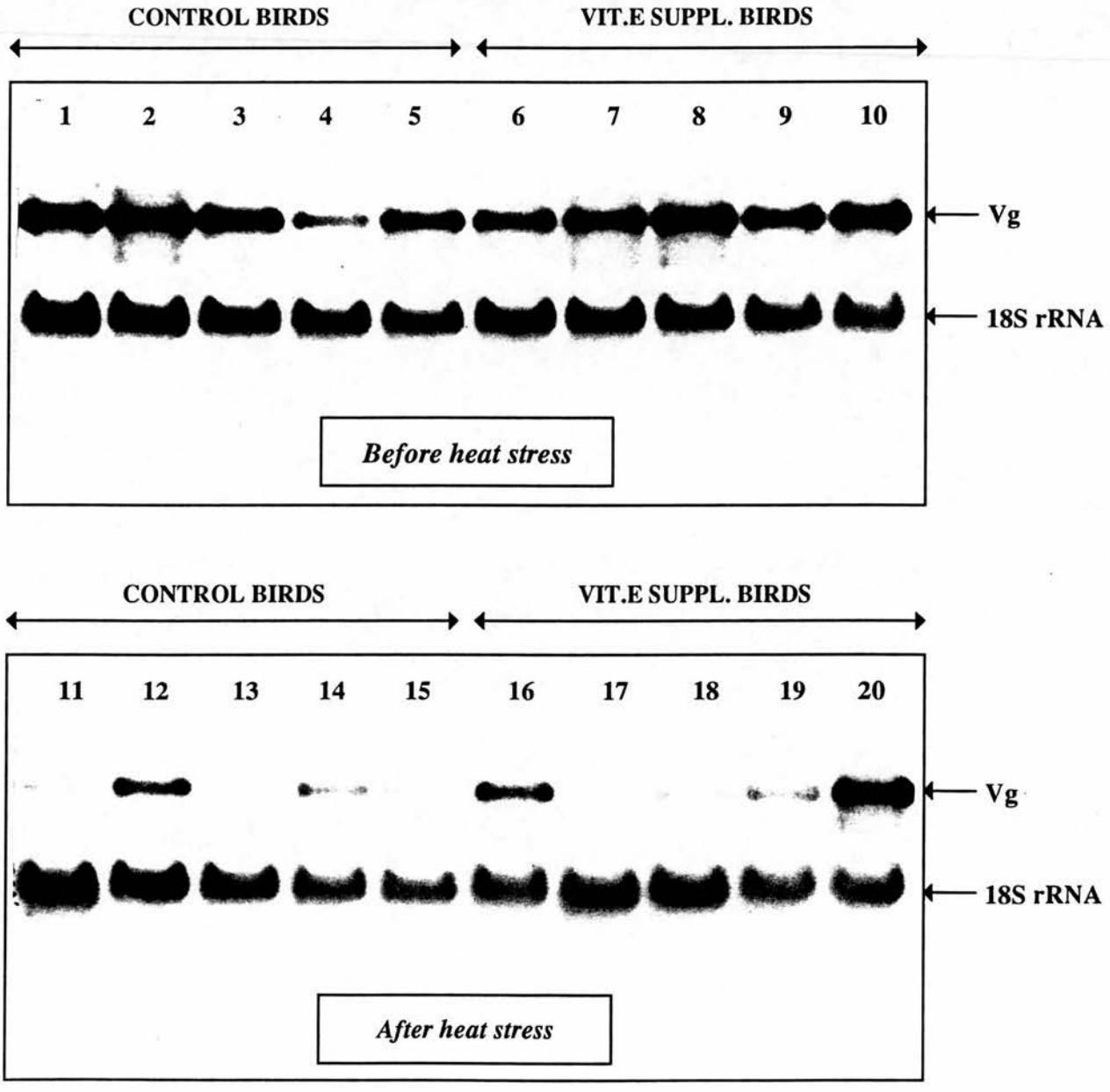
8.1.2.2. Liver vitellogenin mRNA contents

As described in section 8.1.2.3., the appropriate volume corresponding to 10 µl of each RNA sample was loaded into adjacent wells on an agarose gel and electrophoresed. Following transfer of RNA onto a nylon membrane, a ^{32}P-radiolabelled vitellogenin cDNA probe was used for detection of vitellogenin mRNA. The vitellogenin-specific signals were visualised by autoradiography. The strength of each signal is directly proportional to the amount of vitellogenin transcript in the particular sample. However, to allow accurate quantitative comparisons between samples, it was necessary to confirm that equal amounts of each RNA sample were indeed loaded into each lane of the gel. The level of 18S rRNA in each sample, which should not be altered by heat stress or vitamin E supplementation, was used as a loading control. Therefore, the nylon membrane was probed with a ^{32}P-labelled 18S rRNA cDNA probe. The autoradiogram (exposure of 40 minutes) is reproduced in Figure 25.

The signal corresponding to 18S rRNA in both upper and lower panels of Figure 25 showed

equal intensities between lanes confirming that equal amounts of total RNA were analysed. The upper panel of this figure also showed that the vitellogenin-specific signal was not markedly different between samples derived from vitamin E-supplemented (birds 6 to 10) and non-supplemented birds (birds 1 to 5).

Figure 25 : Northern blot showing vitellogenin and 18 S rRNA signals



Phosphorimager analysis of the membrane was performed in order to detect differences in signal intensities which were not discernible by eye and also to provide quantitative data. Presented in the table below (Table 62) are the respective signal intensities expressed as counts per unit time and the vitellogenin-specific signals after correction for minute differences in loading.

Table 62 : Corrected relative counts (expressed as a % of the bird showing the higher corrected count, mean ± S.D.) in birds killed at the end of the thermoneutral period

Bird	Vitellogenin-specific signal (counts per unit time)	Loading control signal (counts per unit time)	Vitellogenin-specific signal (counts per unit time) (corrected for loading differences)
Control group (Group A)			
1	93.42	97.32	**75.16**
2	93.60	100.00	**73.29**
3	77.28	91.78	**65.93**
4	37.81	93.40	**31.69**
5	68.64	82.42	**65.21**
Mean			**62.26 ± 15.78**
Vitamin E-supplemented group (Group B)			
6	65.96	98.86	**52.46**
7	85.78	93.72	**71.67**
8	100.00	92.41	**84.73**
9	63.66	82.44	**60.46**
10	84.56	66.21	**100.00**
Mean			**73.86 ± 16.99**

The mean values for the vitellogenin signal were not significantly different between the two groups of birds suggesting that the steady state level of vitellogenin mRNA was unaffected by vitamin E supplementation during thermoneutral conditions. Taking the 10 birds together, the mean value of the vitellogenin signal was 68.06 ± 17.39 counts per unit time.

The lower panel of Figure 1 shows the vitellogenin-specific signal during heat stress. As was noted before, the signals corresponding to the loading control (18S rRNA) appeared to be similar between samples suggesting equal loading of total RNA. The vitellogenin signals (with the exception of bird 20) showed an overall reduction over birds housed in thermoneutral conditions. It should be emphasised that the upper and lower panels of the blot were produced at the same time and hybridised to the same radiolabelled vitellogenin cDNA probe. Thus the large reduction in vitellogenin signal was a specific effect of heat stress and is not due to differences in transfer of RNA to the nylon membrane or differences in specific activity of the probes. These results, quantified by phosphorimager analysis and corrected to give relative counts, are presented in Table 63.

Table 63 : Corrected relative counts (expressed as a % of the bird showing the higher corrected count, mean ± S.D.) in birds killed at the end of the heat stress period

Bird	Vitellogenin-specific signal (counts per unit time)	Loading control signal (counts per unit time)	Vitellogenin-specific signal (counts per unit time) (corrected for loading differences)
Control group (Group A)			
11	10.72	76.80	**10.93**
12	29.77	77.47	**30.09**
13	10.53	65.18	**12.65**
14	17.63	59.21	**23.31**
15	2.16	51.49	**3.29**
Mean			**16.05 ± 9.49**
Vitamin E-supplemented group (Group B)			
16	38.79	67.64	**44.90**
17	10.87	82.14	**10.37**
18	14.16	88.38	**12.55**
19	21.37	63.76	**26.24**
20	80.30	65.31	**96.27**
Mean			**38.07 ± 31.60**

No significant difference was apparent in amount of vitellogenin mRNA between the two groups of birds. The average corrected relative counts during the heat stress could therefore

also be calculated as the mean of the ten birds together : 27.06 ± 25.80 counts per unit time. If bird 20, which showed a very high signal was discarded, the mean of the 9 birds would be 19.37 ± 12.17 counts per unit time.

The vitellogenin signal obtained during heat stress (even by keeping bird 20 into account) was therefore significantly reduced during heat stress (-60%, $P<0.01$). However, during heat stress and, as was observed under thermoneutral conditions, vitamin E supplementation had no effect on the intensity of the vitellogenin signal.

8.1.2.3. Individual characteristics of the sacrificed birds

8.1.2.3.1. Performance of the birds during the thermoneutral period

The individual performance characteristics of the five birds killed after the first two weeks of the experiment (22°C, 60% R.H.) in each of the control group (Group A) and vitamin E-supplemented group (Group B) (vitamin E-supplemented) are presented in Tables 64 and 65.

Table 64 : Characteristics of the birds killed at the end of the thermoneutral period (mean ± S.D.)

	Total egg mass (g per hen per day)	**Feed intake** (g per hen per day)	**Body weight** (g per hen)
Control group (Group A)			
Bird 1	45.54	121.9	1768
Bird 2	52.53	102.8	1746
Bird 3	54.15	129.4	1914
Bird 4	43.92	110.4	1904
Bird 5	48.98	123.2	1722
Mean	**49.02 ± 3.92**	**117.5 ± 9.6**	**1811 ± 82**
Vitamin E-supplemented group (Group B)			
Bird 6	46.89	119.2	1947
Bird 7	53.07	117.3	1859
Bird 8	56.85	126.1	1848
Bird 9	42.52	92.9	1539
Bird 10	46.44	96.6	1660
Mean	**49.15 ± 5.12**	**110.4 ± 13.2**	**1771 ± 149**

Table 65 : **Plasma and liver concentrations of yolk precursors in birds killed at the end of the thermoneutral period (mean ± S.D.)**

	Plasma Vg-Zn (µg per ml)	Liver Vg-Zn (µg per g)	Ratio plasma/liver Vg-Zn	Liver Triglyceride (mg per g)
Control group (Group A)				
Bird 1	2.65	1.98	1.34	5.45
Bird 2	1.89	1.62	1.17	5.13
Bird 3	1.76	2.42	0.73	5.79
Bird 4	2.80	2.56	1.09	5.29
Bird 5	2.08	1.51	1.38	6.21
Mean	**2.24 ± 0.46**	**2.02 ± 0.42**	**1.14 ± 0.23**	**5.57 ± 0.39**
Vitamin E-supplemented group (Group B)				
Bird 6	2.82	2.26	1.25	5.86
Bird 7	1.75	2.79	0.63	5.18
Bird 8	1.86	2.05	0.91	5.30
Bird 9	2.74	*	*	*
Bird 10	2.62	*	*	*
Mean	**2.36 ± 0.46**	**2.37 ± 0.31**	**0.93 ± 0.25**	**5.47 ± 0.30**
OVERALL MEAN (Mean of control and vitamin E-supplemented groups)				
	2.30 ± 0.44	**2.15 ± 0.42**	**1.06 ± 0.26**	**5.53 ± 0.36**

* : samples lost during centrifugation

The average egg production, feed intake and body weight were similar in the two groups of birds and close to those presented in Table 60. Also, no effect of vitamin E supplementation was observed on the concentrations of egg yolk precursors, plasma and liver vitellogenin-bound zinc and liver triglyceride. The relative concentration of plasma vitellogenin-bound zinc to that of liver vitellogenin-bound zinc can be denoted by a ratio V where :

$$V = \frac{\text{plasma Vg-Zn concentration}}{\text{liver Vg-Zn concentration}}$$

In other words, as V represents the relative proportion of vitellogenin-bound zinc present in the plasma to the one present in the liver, V can be considered as an indicator of the intensity of "transfer" of vitellogenin from the liver to the plasma. In thermoneutral conditions, V was

similar in both groups and close to a value of 1 showing that both the amounts of vitellogenin-bound zinc present in the plasma and in the liver were similar or "in equilibrium".

8.1.2.3.2. Performance of the birds during the heat stress period

The individual performance characteristics of the five birds killed after the two weeks of the exposure to heat stress (32 to 35°C, 70% R.H.) in each of the control group (Group A) and vitamin E-supplemented group (Group B) (vitamin E-supplemented) are presented in Tables 66 and 67.

Table 66 : Characteristics of the birds killed at the end of the heat stress period (mean ± S.D.)

	Total egg mass (g per hen per day)	**Feed intake** (g per hen per day)	**Body weight** (g per hen)
Control group (Group A)			
Bird 11	46.12	68.2	1560
Bird 12	44.71	65.2	1560
Bird 13	36.43	46.9	1320
Bird 14	24.12	52.3	1690
Bird 15	16.00	41.8	1310
Mean	**33.48 ± 11.73 a**	**54.9 ± 10.2 a**	**1488 ± 149**
Vitamin E-supplemented group (Group B)			
Bird 16	49.79	70.9	1530
Bird 17	48.57	61.4	1520
Bird 18	47.53	61.1	1490
Bird 19	46.71	66.8	1600
Bird 20	45.96	72.7	1550
Mean	**47.71 ± 1.35 b**	**66.6 ± 4.7 b**	**1538 ± 37**

Within a column, the values indicated by different letters are statistically different ($p<0.05$)

During the heat stress period, the total egg mass of killed non-supplemented birds was 30% ($P<0.05$) lower than that of the killed vitamin E-supplemented birds. The plasma Vg-Zn concentration was significantly ($P<0.02$) lower in both groups than pre-stress values (cf. Table 65). However, the difference between the two stressed groups failed to reach significance

(because of the low number of replications), although the vitamin E treatment increased the concentration of vitellogenin in the plasma by the same magnitude (+30%) as it increased the total egg mass produced during heat stress.

Table 67 : Plasma and liver concentrations of egg yolk precursors in birds killed at the end of the heat stress period (mean ± S.D.)

	Plasma Vg-Zn (µg per ml)	Liver Vg-Zn (µg per g)	Ratio plasma/liver Vg-Zn	Liver Triglyceride (mg per g)
Control group (Group A)				
Bird 11	2.01	3.77	0.53	2.58
Bird 12	1.29	4.20	0.31	3.67
Bird 13	1.28	4.85	0.26	3.07
Bird 14	0.94	3.92	0.24	3.32
Bird 15	0.31	2.25	0.14	3.38
Mean	**1.16 ± 0.55**	**3.80 ± 0.86 a**	**0.30 ± 0.13 a**	**3.20 ± 0.37**
Vitamin E-supplemented group (Group B)				
Bird 16	1.10	1.16	0.95	3.17
Bird 17	1.67	3.61	0.46	3.17
Bird 18	2.03	3.22	0.63	3.40
Bird 19	1.86	2.45	0.76	3.77
Bird 20	1.57	1.46	1.08	3.62
Mean	**1.65 ± 0.31**	**2.38 ± 0.95 b**	**0.78 ± 0.22 b**	**3.43 ± 0.24**

Within a column, the values indicated by different letters are statistically different ($p<0.05$)

The liver Vg-Zn concentration was also affected by heat stress but in a different manner. In non-supplemented birds, it was markedly increased by 77% ($P<0.01$) during heat stress compared to its level pre-stress : 3.80 ± 0.86 µg per g. However, for the supplemented birds, this increase was not observed. The Vg-Zn concentration after heat stress remained similar to the pre-stress value : 2.38 ± 0.95 µg per g versus 2.15 ± 0.42 µg per g respectively. During heat stress, Vg-Zn concentration in the liver was therefore markedly higher in the control group than in the vitamin E-supplemented one (+60%, $P<0.05$).

The concentration of triglyceride in the liver was significantly depressed in both the non-supplemented and vitamin E-supplemented groups and the magnitudes of these decreases were

similar (-42% and -38% respectively, $P<0.01$) compared to the concentrations observed during the thermoneutral period.

The ratio V (section 8.1.3.1.) was significantly reduced ($P<0.01$) during heat stress in the non-supplemented group to reach a value of 0.30 ± 0.13. During thermoneutral conditions, V was 1.06 ± 0.26. In the supplemented birds, V was also reduced (0.78 ± 0.22) but was not significantly different from its level in thermoneutral conditions. As a consequence, during heat stress, V was significantly higher in vitamin E-supplemented birds than in non-supplemented birds (+126%, $P<0.01$).

8.1.3. Discussion

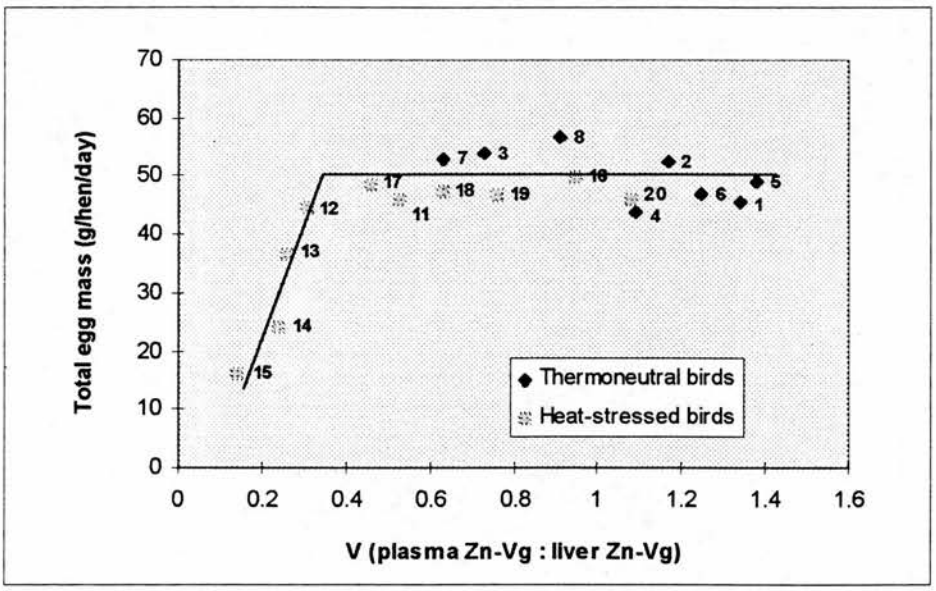
Experiment 8 was designed to investigate the effect of heat stress and vitamin E on the major egg yolk precursor protein, vitellogenin. The latter is synthesised in the liver following transcription of the vitellogenin gene. As a measure of vitellogenesis, the steady state level of vitellogenin mRNA was examined. The amount of vitellogenin transcript was found to be significantly ($P<0.01$) reduced during heat stress. This could be due to a reduction in the level of transcription of the vitellogenin gene during heat stress or to an increased destruction/cleavage of the vitellogenin message. However, the amount of vitellogenin transcript was found to be unaffected by the vitamin E supplementation. Although this result could have been expected in thermoneutral conditions as both vitamin E-supplemented and non-supplemented birds showed identical egg production and total egg mass output, the absence of significant effect of vitamin E during heat stress was more surprising as supplemented birds showed higher egg production performances than non-supplemented birds. As a result, the increase in egg production observed in vitamin E-supplemented compared to non-supplemented birds during heat stress cannot be explained by the presence of greater amounts of vitellogenin mRNA in the liver of supplemented birds.

Further work was therefore carried out to determine whether the concentration of the vitellogenin protein in the liver responded in a similar way to heat stress and vitamin E treatments. This work demonstrated that, during the thermoneutral period, the liver vitellogenin protein concentration was similar in both vitamin E-supplemented and non-supplemented birds. However, during heat stress, the liver vitellogenin protein concentration was found to be markedly elevated by 77% ($P<0.01$) in control or **non-supplemented birds** compared to pre-heat stress levels. In contrast, this increase was not seen in birds which

received the vitamin E-supplemented diet (Table 67).

The increase in liver vitellogenin concentration in stressed, unsupplemented birds was rather unexpected as other parameters, such as egg production, amount of vitellogenin mRNA in the liver and concentration of vitellogenin-bound zinc in the plasma, were depressed in these birds. During heat stress, as the concentration of vitellogenin-bound zinc was increased in the liver but decreased in the plasma of control birds, V was markedly reduced in the non-supplemented birds (-72%, $P<0.01$) and maintained to a value closer to the pre-stress value in supplemented birds (-26%, NS). To represent the relation between V and total egg mass, the regression curve between these characteristics was represented (Figure 26).

Figure 26 : Relationship between the ratio V and total egg mass.



This regression curve revealed two interesting points. Firstly, during the thermoneutral period, there was no apparent relationship between V and total egg mass. Birds 1 to 8 showed ratio values ranging between 0.6 and 1.4 and for all these birds the total egg mass was independent of V and ranged from 44 to 57 g per hen per day. Furthermore, both V and total egg mass were similar in the non-supplemented and supplemented birds.

Secondly, during the heat stress period, a marked difference appeared between the non-supplemented and supplemented birds. For the non-supplemented birds (birds 11 to 15), V values ranged between 0.1 and 0.5 and a clear relationship appeared between V and total egg mass (ranging between 16 and 46 g per hen per day). The lower V, the lower the total egg

mass. It should also be pointed out, at this stage, that for the birds showing low V values (birds 13, 14 and 15), food consumption was relatively low (46.9 g, 52.3 g and 41.8 g/day respectively). This could, at least partially, explain in the reduction of egg production in these birds.

In the supplemented birds (birds 16 to 20), V values were higher (ranging between 0.5 and 1.1) and did not correlate with the total egg mass (ranging between 46 and 50 g per hen per day). As food intake was also higher in these birds, it is possible that vitamin E affected egg production by initially stimulating food intake. However other studies would be required to investigate the interaction between vitamin E supplementation and food intake during stress conditions.

To summarise, low V values occurred during heat stress for the non-supplemented birds and correlated directly with low total egg mass. The increase in liver zinc-vitellogenin concentration and corresponding decrease in plasma concentration in these birds therefore appeared to result in a lower egg output. This observation is of profound physiological significance. The results suggest that heat stress may interfere with the export of the vitellogenin protein from the hepatocyte following translation of vitellogenin mRNA. Vitamin E supplementation appeared to alleviate the problem and reduced the accumulation of the newly synthesised vitellogenin protein in the liver. A possible explanation for the role of vitamin E in this process can be proposed by referring to the antioxidant properties of vitamin E.

Stress has been known for a long time to increase the amount of free-radicals in cells (section 2.2.5.). As hepatocytes are, in laying hens, very active cells (due to the demand imposed by egg production), they are also very susceptible to stress. Supplementation with vitamin E, which results in an increase in the liver concentration of vitamin E (Chapter 4), may impede the formation or the effects of free radicals in the hepatocytes. As a result, the detrimental effect of free radicals on the cellular lipids (lipid peroxidation) occurring during heat stress may be alleviated. This would allow a greater stability of the membranes, including the organelle membranes, and possibly a reduced effect of heat stress on the export of vitellogenin from the hepatocytes into the circulation. This impaired export was not observed for the triglyceride in the liver as their concentration was significantly depressed during heat stress but in a similar extent in both non-supplemented and vitamin E-supplemented birds. The difference of response between the zinc-vitellogenin and triglyceride concentrations to the vitamin E

supplementation may be due to the difference in the export pathways of vitellogenin and triglyceride from the liver.

8.2. Conclusion

Experiment 8 demonstrated that the amount of vitellogenin transcript, although significantly ($P<0.01$) reduced during heat stress, was not affected by vitamin E supplementation. Indeed, regardless of the ambient temperature, vitamin E had no effect on the steady state level of the vitellogenin message. However, vitamin E was found to have a marked effect on the concentration of the vitellogenin protein in the liver. During heat stress, the liver concentration of vitellogenin was significantly increased (+77%, $P<0.01$) in birds fed a non-vitamin E supplemented diet compared to the concentration observed in thermoneutral conditions. In vitamin E-supplemented birds, this increase did not occur and both the plasma concentration of vitellogenin and egg production were improved compared to the non-supplemented birds. These results suggested that the export of vitellogenin from the liver to the circulation may have been altered during heat stress in non-vitamin E supplemented birds. However, in birds fed a vitamin E-supplemented diet, this effect was, at least in part, alleviated and may explain the greater concentration of vitellogenin in the circulation and improved egg production during heat stress.

In the following chapter, an experiment was carried out to visualise the distribution of the vitellogenin protein in the hepatocytes of vitamin E-supplemented and non-supplemented birds during heat stress. This was done using a vitellogenin-specific antibody and the techniques of immunogold labelling and electron microscopy. To test the reproducibility and improve the significance of the result obtained in this chapter, greater number of birds were used.

Chapter 9

Effects of chronic heat stress and vitamin E on vitellogenin localisation in hepatocytes

The results obtained in experiment 8 confirmed the findings of Utomo *et al.* (1994) who showed that, during heat stress, plasma vitellogenin concentration was significantly reduced in birds fed a control diet and improved in birds fed a vitamin E-supplemented diet. Experiment 8 also demonstrated that, during heat stress, despite the fact that the amount of vitellogenin mRNA was reduced in the hepatocytes of both groups of birds compared to thermoneutral conditions, the concentration of vitellogenin protein in the liver was markedly increased (+77%, $P<0.01$) in birds fed the control diet (10 mg vitamin E per kg diet) but maintained at a normal level in the birds fed the vitamin E-supplemented diet (300 mg vitamin E per kg diet).

As only a small number of birds were studied in experiment 8 (5 birds in each of the control and vitamin E-supplemented groups), another experiment was carried out (Experiment 9) to validate this result on a larger number of birds. An attempt to visualise the increase, or accumulation, of the vitellogenin protein during heat stress in control birds was also made using electron microscopy and antibodies raised against chicken vitellogenin.

9.1. Experiment 9

The objective of this experiment was to determine whether the results obtained in experiment 8 could be reproduced with a larger number of birds. In particular, experiment 9 was designed to verify whether birds exposed to a chronic period of heat and fed a control diet (10 mg vitamin E per kg diet) experienced an increase in their liver concentration of vitellogenin protein. Another objective was to determine whether vitamin E alleviated this effect. And finally, this experiment was designed to investigate the effects of heat stress and vitamin E on the subcellular localisation of vitellogenin within the hepatocytes. The latter may help to explain

how vitamin E increased egg production in heat-stressed laying hens.

9.1.1. Determination of the number of birds required

To determine the minimal number of birds required in experiment 9 to give a high probability of demonstrating a significant difference between non-supplemented and vitamin E-supplemented birds, calculations were made taking into account the findings of Cochran and Cox (1966). The minimal number of replications, r, required for a given probability, P, of demonstrating a significant difference between two groups, or treatments, is given by the following equation :

$$r \geq 2 \, (\sigma / \delta)^2 \, (t_1 + t_2)^2$$

where δ = true difference that is desired to detect

σ = true standard error per unit

t_1 = significant value of t in the test of significance

t_2 = value of t in the ordinary table corresponding to 2 (1 - P)

Both the difference, δ, in the zinc-vitellogenin concentration between the non-supplemented and supplemented groups and the coefficient of variation, σ, of the concentration obtained in each tested group were estimated from the results obtained in experiment 8. These were approximately 60% and 40% respectively. As experiment 8 also demonstrated that the non-supplemented birds showed a greater concentration of vitellogenin protein in the liver than the vitamin E-supplemented birds, a one-tailed test was used. To have a chance of 4 in 5 that a significant result was obtained and to make this test significant at 5%, the t_1 value was the value for probability 10%, while t_2 was the value for probability (2 (1 - 0.80)) or 40%. Furthermore, as two treatments were tested (non-supplemented versus supplemented), the number of degrees of freedom for the estimate of error, n, was calculated from the following equation :

$$n = (2-1) \, (r-1) = (r-1) \text{ d.f.}$$

In order to start the calculation, some guess had to be made about the value of n. It did not matter greatly if the guess was inaccurate (see below) and n = 30 was tried. For this,

$t_1 = 1.697$; $t_2 = 0.854$; so that $(t_1 + t_2) = 2.551$

Hence,

$$r \geq 2\ (40 / 60)^2\ (2.551)^2 = 5.78$$

Consequently our second trial value was r = 6. Since this provides only 5 instead of 30 d.f., we repeated the calculation to verify whether 6 replications were sufficient. For 5 d.f., $(t_1 + t_2)$ is 2.935. Thus,

$$2\ (\sigma / \delta)^2\ (t_1 + t_2)^2 = 2\ (40 / 60)^2\ (2.935)^2 = 7.66$$

Since this is greater than 6, we concluded that 6 replications were not quite enough, and that 8 is the smallest number of replications that would satisfy the conditions. We could be sure without further computation that 8 satisfied the conditions, because the right-hand side of the inequality always decreases when we increase r.

As these calculations were based on the difference, δ, and coefficient of variation, σ, obtained in the previous experiment, and as the difference obtained in the previous trial was quite high (60%), the calculations were repeated by taking a new assumption for the value of δ. To increase the margin of security, we supposed that the difference between the treatment was 50% instead of 60%. In this case, and with a chance of 4 in 5 that a significant result is obtained, the new number of replication required was calculated and in order to start the calculation, n = 30 was tried. For this, $(t_1 + t_2) = 2.551$. Hence,

$$r \geq 2\ (40 / 50)^2\ (2.551)^2 = 8.33$$

Consequently the second trial value was r = 9. Since this provided only 8 instead of 30 d.f., the calculation were repeated to verify whether 9 replications are sufficient. For 8 d.f., $(t_1 + t_2)$ is 2.749. Thus,

$$2\ (\sigma / \delta)^2\ (t_1 + t_2)^2 = 2\ (40 / 50)^2\ (2.749)^2 = 9.67$$

Therefore the smallest number of replications that would satisfy the conditions was 10. To be sure to have enough birds in the next experiment, and that the determination of the birds required was not based on a too optimistic δ value, a minimal number of 10 birds per group were used during the heat stress period in experiment 9.

9.1.2. Materials and methods

9.1.2.1. Selection of the birds and husbandry

Twenty-six young Isa Brown birds aged 20 weeks were housed in a room under thermoneutral conditions (22°C, 60% R.H.), and fed a standard diet containing 10 mg vitamin E per kg diet for 3 days (adaptation period). On the third day, the birds were individually weighed and paired according to their body weight. Two birds showing a close body weight formed a pair, one bird receiving a control diet (10 mg α-tocopherol per kg diet), the other receiving a vitamin E supplemented diet (300 mg α-tocopherol per kg diet). The average body weights were thus similar in both control (Group A) and vitamin E-supplemented (Group B) groups and were respectively 1775 ± 166 g and 1797 ± 160 g.

The birds were fed these diets for 14 days and the temperature and humidity were maintained at 22°C, 60% R.H. Then three pairs of birds (6 birds) were blood sampled, and killed as described in Chapter 5. As soon as the birds were killed, their liver was collected to minimise degradation of vitellogenin and processed for determination of zinc-vitellogenin concentration (section 9.1.2.2.) and observation by electron microscopy (section 9.1.2.3.). The ambient temperature of the room was then increased to 34°C for three further weeks and the relative humidity was increased to 70%. After this three-week period, the remaining ten pairs (20 birds) were blood sampled, killed and livers were removed and processed as described earlier. The liver concentration of zinc-vitellogenin was determined on all the birds and the observation by electron microscopy was carried out on three pairs of birds (6 birds).

9.1.2.2. Processing of the samples for determination of liver vitellogenin concentration

As soon as a bird was killed, a sample of approximately 1.3 g of liver was collected, placed in 4 ml of 0.9% saline and processed and assayed for zinc-vitellogenin concentration as described in section 8.1.1.3.

9.1.2.3. Processing of the samples for immunoelectron microscopical observation

9.1.2.3.1. Description of the method

The immunocytochemistry technique is based on the fact that the body reacts to foreign protein substances, antigens, by producing specific substances, antibodies, which combine with and

inactivate the antigens. To localise an antigen by immunocytochemistry and using electron microscopy, there are both direct and indirect methods. The sections of a tissue suspected of containing an antigen can be incubated with a specific antibody which has been labelled with a tag, so that the antibody combines with the antigen and can be detected directly by electron microscopy. However, as this method is not very sensitive, an indirect method was used. The tissue sections were initially exposed to unlabelled vitellogenin antibody, resulting in the formation of an invisible antigen-antibody complex. A secondary labelled antibody to the primary antibody was then applied to the tissue sections. This labelled antibody combined with the unoccupied antigenic sites in the primary immune complex and which is thus made visible.

This indirect method was used in experiment 9. As anti-chicken vitellogenin antibody is not available commercially, a sample of this antibody, raised in rabbit, was kindly provided by Dr. D. Williams from the State University of New York (Unpublished). The secondary anti-rabbit IgG antibody, raised in goat and labelled with 15 nm gold particles, was purchased from Agar Scientific Ltd. (Stansted, U.K.).

9.1.2.3.2. Tissue preparation

As soon as the liver was extracted from the bird, small pieces (1 mm^3) were fixed by immersion at 4°C for 1 hour in a mixture of 0.175 M cacodylate and 1% osmium tetroxide, pH 7.4. After fixation, the tissue blocks were washed and left overnight in phosphate buffer containing 0.22 M sodium dihydrogen orthophosphate, 0.19 M sodium hydroxide and 0.06 M glucose. The next morning, the blocks were dehydrated by immersion through graded ethanols, 50% ethanol for 5 and 10 minutes, 95% ethanol for 2 x 20 minutes and 100% ethanol for 2 x 25 minutes. Once dehydrated, the blocks were immersed in filtered inhibisol (epoxypropane) for 2 x 5 minutes. Then they were immersed and left overnight at room temperature in a solution containing 50% inhibisol and 50% araldite. The next morning, the blocks were embedded in pure araldite and left for 48 hours at room temperature. Ultrathin sections (approximately 300 Å) were cut with a glass knife, mounted on 400-mesh nickel grids purchased from Agar Scientific Ltd. (Stansted, U.K.).

9.1.2.3.3. Electron microscopical post-embedding techniques

All labelling procedures were carried out at room temperature and essentially followed the method recommended by the Royal Postgraduate Medical School of the University of London

(1993). The different incubations were carried out on pieces of fresh laboratory film and in a very clean environment and all buffers and washing solutions were microfiltered (0.22 μm pore size). The sections, mounted on nickel grids, were placed on drops of the washing or staining solutions, drained on to fibre-free absorbent paper between each solution but not allowed to dry during the process.

The labelling procedure was performed as follows :

1. The sections were incubated in 0.1% sodium periodate for 10 minutes to permeabilise the araldite resin.
2. The sections were then drained and washed thoroughly in double distilled water for 3 x 1 minute.
3. The sections were drained and incubated for 30 minutes in normal goat serum, diluted 1/30 in antiserum diluent. The antiserum diluent contained 0.01 M phosphate-buffered saline, pH 7.4, 0.15 M NaCl, 0.1% bovine serum albumin (BSA, from Sigma, fraction V, globulin free) and 0.01% sodium azide.
4. The normal goat serum was drained from the grids and the grids were placed in the primary antibody, diluted 1/100 in the antiserum diluent described earlier. A grid, used as a control, was not placed in the primary antibody but left in normal goat serum. The grids were incubated in these solutions for 1 hour.
5. All the grids were drained and washed thoroughly in 0.05 M Tris HCl buffer, Tris-I, pH 7.4, for 3 x 1 minute.
6. The grids were drained and washed thoroughly in 0.05 M Tris HCl buffer, Tris-II, pH 7.4, containing 0.1% BSA for 3 x 1 minute.
7. The grids were drained and placed in 0.05 M Tris HCl buffer, Tris-III, pH 8.2, containing 1.0% BSA for 15 minutes.
8. After centrifugation of the secondary gold labelled antibody for 5 minutes at 700 g to remove aggregates, the grids were placed in drops of this secondary antibody, diluted 1/20 in Tris-II buffer, for 1 hour.
9. The grids were drained and washed thoroughly in Tris-II buffer for 3 x 1 minute.
10. The grids were drained and washed thoroughly in Tris-I buffer for 3 x 1 minute.

11. The grids were drained and washed thoroughly in double distilled water for 3 x 1 minute.

12. The grids were drained and counterstained for conventional electron microscopy. Firstly, they were placed in a solution containing 2% uranyl acetate and 50% ethanol for 3 minutes, then they were washed thoroughly in 10% methanol, drained and placed in lead citrate for 4 minutes (cover from air) and finally washed thoroughly in double distilled water and air dried.

The sections were then examined in a CM 10 transmission electron microscope (Philips) at 100 kV and the photographs were taken with a magnification of 40,500.

9.1.3. Results

9.1.3.1. Performance of the birds and liver zinc-vitellogenin concentration

9.1.3.1.1. During the thermoneutral period

The performance characteristics (egg production, food intake and body weight) and liver zinc-vitellogenin concentration of the birds over the thermoneutral period (22°C, 60% R.H.) are presented in table 68.

Table 68 : Characteristics of the birds during the thermoneutral period (mean ± S.D.).

	Group A (n=13)	**Group B (n=13)**
Egg production (No eggs/hen/day x 100)	77.51 ± 24.96	76.92 ± 25.77
Mean egg weight (g/egg)	53.41 ± 4.03	51.00 ± 2.30
Total egg mass (g/hen/day)	41.35 ± 13.61	39.27 ± 13.37
Feed intake (g/hen/day)	100.7 ± 17.5	102.6 ± 22.2
Body weight (g/hen)	1746 ± 202	1758 ± 162
Liver Zn-Vg concentration (µg/g)	**2.34 ± 0.50 (n = 3)**	**2.34 ± 1.14 (n = 3)**

All these characteristics were similar in both non-supplemented and vitamin E-supplemented

groups. The concentration of zinc-vitellogenin in the liver, determined on the three pairs of birds killed at the end of the thermoneutral period, was also identical in both groups of birds and similar to the concentrations observed in experiment 8. Indeed, in the present experiment, an average value of 2.34 ± 0.88 µg per g was obtained versus 2.15 ± 0.42 µg per g in experiment 8.

9.1.3.1.2. During the heat stress period

Egg production, feed intake, body weight and liver zinc-vitellogenin concentration of the birds killed at the end of the heat stress period (34°C, 70% R.H.) are presented in Table 69.

Table 69 : Characteristics of the birds during the heat stress period (mean ± S.D.).

	Group A (n=10)	**Group B (n=10)**
Egg production (No eggs/hen/day x 100)	55.73 ± 11.75	66.39 ± 14.06
Mean egg weight (g/egg)	52.25 ± 4.46	48.11 ± 2.54
Total egg mass (g/hen/day)	28.86 ± 5.18	32.11 ± 7.48
Feed intake (g/hen/day)	54.2 ± 9.1	59.2 ± 12.2
Body weight (g/hen)	1496 ± 155	1479 ± 144
Liver Zn-Vg concentration (µg/g)	**3.81 ± 0.95 a (n = 10)**	**2.50 ± 1.07 b (n = 10)**

Within a line, the values affected by different letters are statistically different (P<0.05).

During heat stress, egg production, food intake and body weight were depressed in both groups of birds compared to pre-stress values. This magnitude of the decrease in egg production was reduced in the group fed the vitamin E-supplemented diet and egg production was improved by 10.7% (P<0.09) in the supplemented compared to the control group.

The concentration of zinc-vitellogenin in the liver was also affected during the stress period, especially in the control group. As was observed in experiment 8, the liver concentration of zinc-vitellogenin was markedly increased compared to the pre-stress value in the control group and reached 3.81 ± 0.95 µg per g instead of 2.34 ± 0.88 µg per g before the stress (+63%,

P<0.01). In the vitamin E-supplemented group, this heat stress-induced increase did not occur and the liver concentration of zinc-vitellogenin was maintained at a similar value to before the stress (2.50 ± 1.07 µg per g). The concentration of zinc-vitellogenin in the liver was therefore significantly higher in the control group (+52%, P<0.01) than in the vitamin E-supplemented group during the period of stress. These results therefore confirmed those obtained in experiment 8, in which liver concentrations of zinc-vitellogenin of 3.80 ± 0.86 µg per g and 2.38 ± 0.95 µg per g were obtained in the control and vitamin E-supplemented groups respectively during the stress period.

9.1.3.2. Intracellular localisation of vitellogenin

9.1.3.2.1. During the thermoneutral period

Liver sections were examined from a representative bird in the control group (bird A) and in the vitamin E-supplemented group (bird B). These two birds were chosen so that their liver concentration of zinc-vitellogenin reflected as closely as possible the average concentration of vitellogenin in their respective groups. Bird A showed a zinc-vitellogenin concentration of 2.46 µg per g of liver and bird B showed a zinc-vitellogenin of 2.31 µg per g of liver. For each of these two birds, four sections were examined and these sections differed in the type of post-embedding treatment they were subjected to (section 9.1.2.3.3.). The first two sections (section 1 and 2) were treated with the primary antibody and the other two sections (section 3 and 4) were not and were therefore used as controls. The photographs of these sections are given in Figure 27 (bird A, sections A1 to A4) and in Figure 28 (bird B, sections B1 to B4).

The photographs of the samples used as controls (lower panels of Figures 27 and 28) confirmed that the secondary antibody, labelled with particles of gold, did not bind proteins non-specifically. The photographs of the upper panels of Figures 27 and 28 revealed the presence of gold particles and therefore suggested that the primary antibody had hybridised. As expected, no difference in the intensity of labelling was apparent between the control and vitamin E-supplemented bird. However, in both photographs, the intensity of the labelling was very low compared to this observed by Kami and Stoward (1985) and gold particles were found in organelles which should not contain vitellogenin. This may be due to the fact that the primary antibody had a low sensitivity and was not specific to vitellogenin.

Figure 27 : Hepatocytes from bird A (fed a diet containing 10 mg vitamin E/kg diet and housed in thermoneutral conditions, 22°C, 60% R.H.) fixed with a mixture of 0.175 M cacodylate and 1% osmium tetroxide, embedded in araldite and labelled for vitellogenin with the immunogold technique.

The upper panels (sections A1 and A2) of the figure correspond to sections which were treated with the primary antibody and the lower panels (sections A3 and A4) to sections which were not treated with this antibody and therefore used as controls.

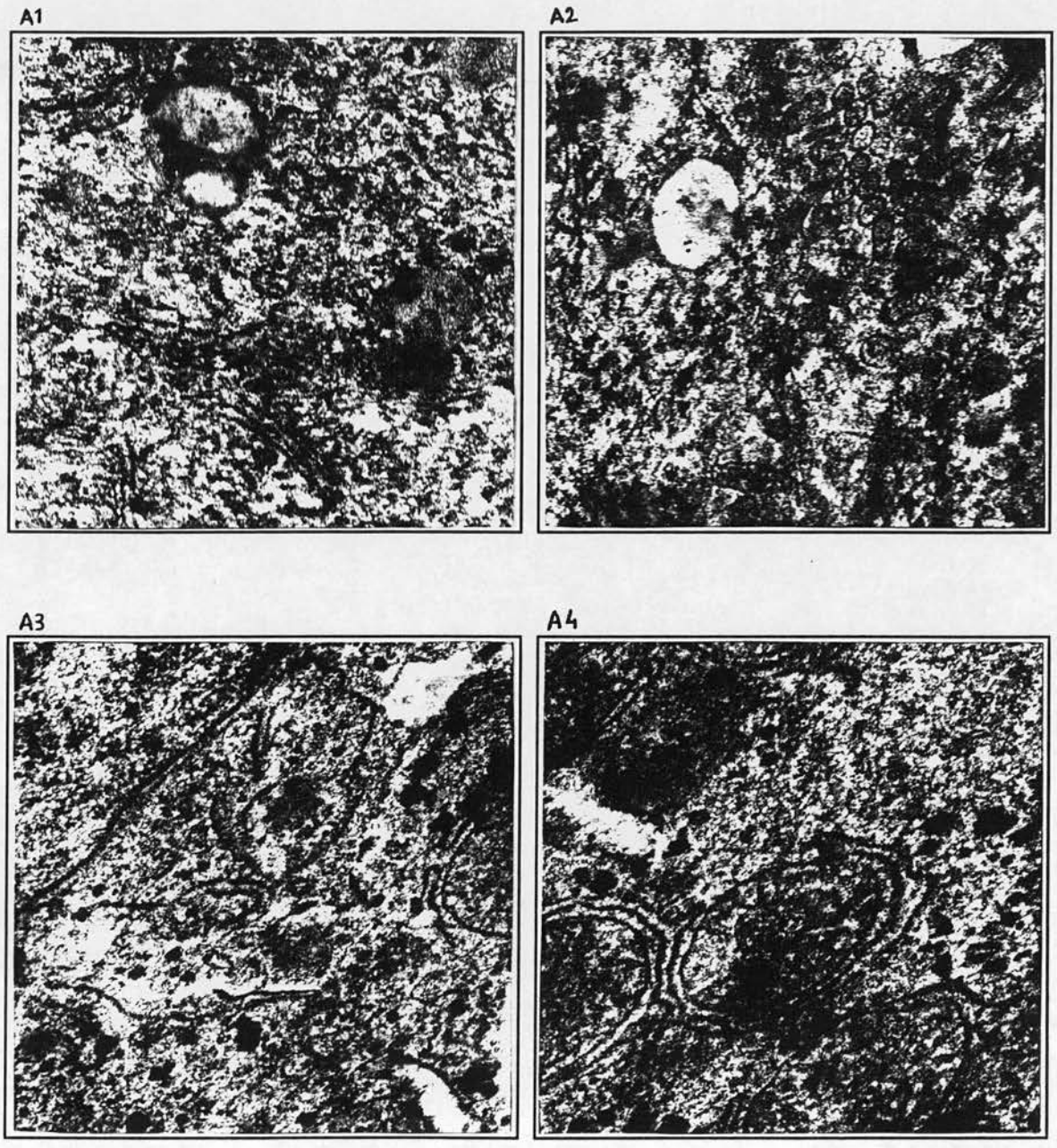
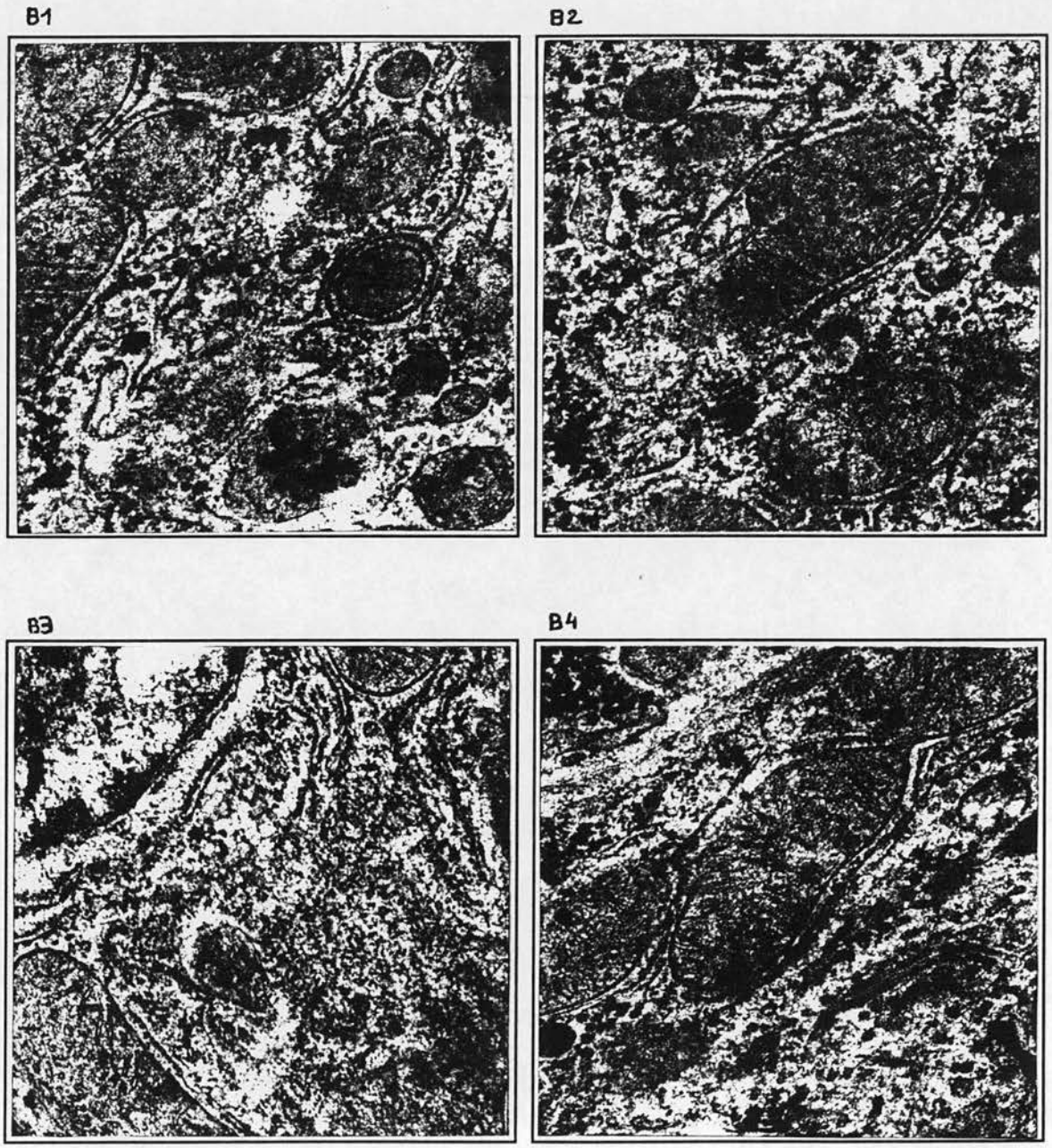


Figure 28 : Hepatocytes from bird B (fed a diet containing 300 mg vitamin E/kg diet and housed in thermoneutral conditions, 22°C, 60% R.H.) fixed with a mixture of 0.175 M cacodylate and 1% osmium tetroxide, embedded in araldite and labelled for vitellogenin with the immunogold technique.

The upper panels (sections B1 and B2) of the figure correspond to sections which were treated with the primary antibody and the lower panels (sections B3 and B4) to sections which were not treated with this antibody and therefore used as controls.



9.1.3.2.2. During the heat stress period

Similarly as in section 9.1.3.2.1., liver sections were examined from a representative bird in the control group (bird C) and in the vitamin E-supplemented group (bird D). These two birds were chosen so that their liver concentration of zinc-vitellogenin reflected as closely as possible the average concentration of vitellogenin in their respective groups. Bird C showed a zinc-vitellogenin concentration of 4.08 µg per g of liver and bird D showed a zinc-vitellogenin of 3.46 µg per g of liver. This latter value was quite high compared to the average value observed in the vitamin E supplemented group, but unfortunately, none of the two other birds killed in this group showed lower zinc-vitellogenin concentration. For each bird, four sections were examined, sections 1 and 2 were treated with the primary antibody and sections 3 and 4 were not. The photographs of these sections are presented in Figure 29 (bird C, sections 1 to 4) and in Figure 30 (bird D, sections 1 to 4).

As was observed in the previous section, the photographs of the samples used as controls (lower panels of Figures 29 and 30) revealed that the secondary antibody, labelled with particles of gold, did not bind proteins non-specifically. The upper panels of Figures 29 and 30 showed that the hybridisation of the primary antibody was low and was possibly non-specific in certain organelles.

Figure 29 : Hepatocytes from bird C (fed a diet containing 10 mg vitamin E/kg diet and housed in heat stress conditions, 34°C, 70% R.H.) fixed with a mixture of 0.175 M cacodylate and 1% osmium tetroxide, embedded in araldite and labelled for vitellogenin with the immunogold technique.

The upper panels (sections C1 and C2) of the figure correspond to sections which were treated with the primary antibody and the lower panels (sections C3 and C4) to sections which were not treated with this antibody and therefore used as controls.

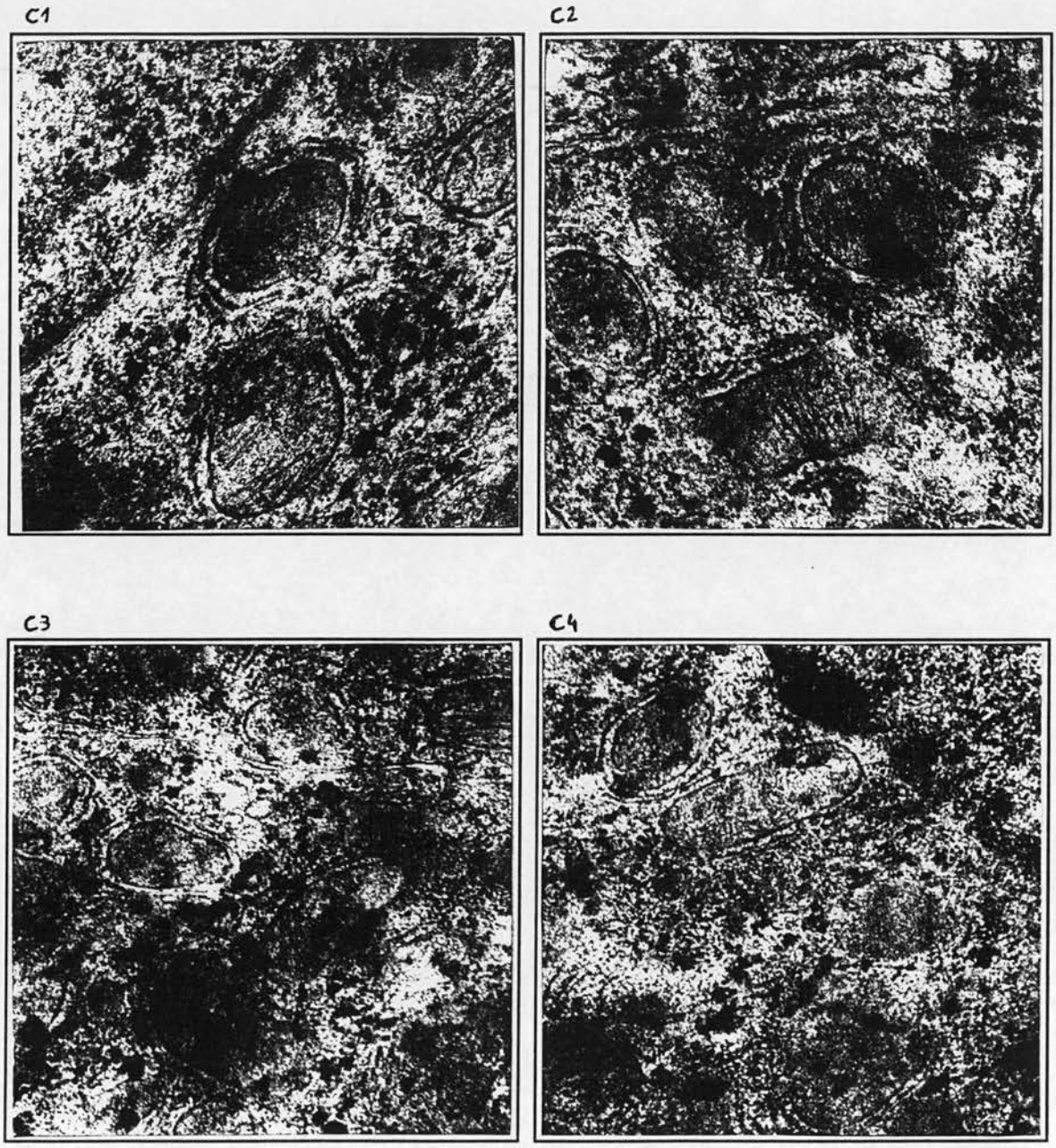
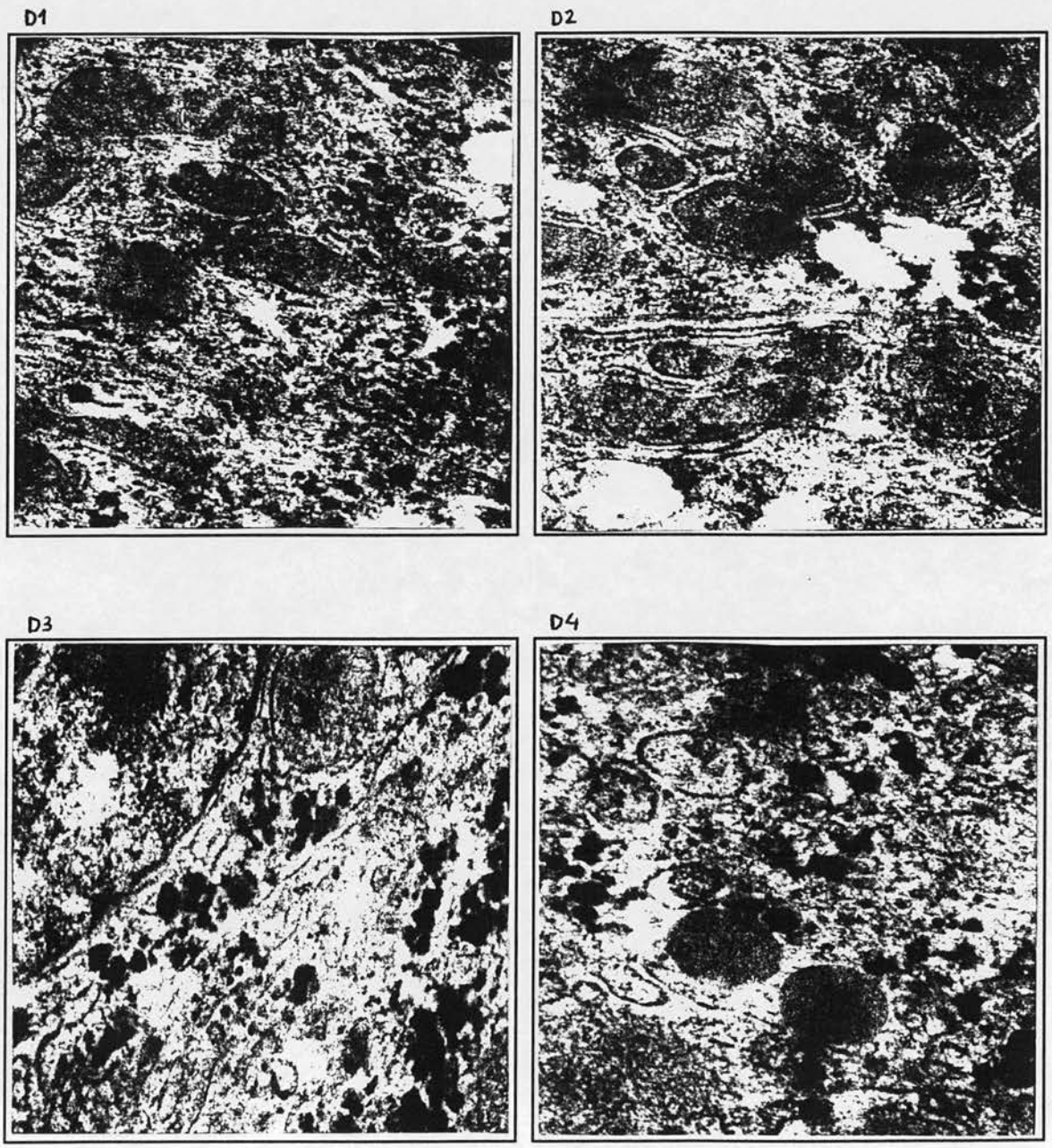


Figure 30 : Hepatocytes from bird D (fed a diet containing 300 mg vitamin E/kg diet and housed in heat stress conditions, 34°C, 70% R.H.) fixed with a mixture of 0.175 M cacodylate and 1% osmium tetroxide, embedded in araldite and labelled for vitellogenin with the immunogold technique.

The upper panels (sections D1 and D2) of the figure correspond to sections which were treated with the primary antibody and the lower panels (sections D3 and D4) to sections which were not treated with this antibody and therefore used as controls.



9.1.4. Discussion

The determination of the concentration of zinc-vitellogenin in the liver during and after the period of heat stress confirmed the results obtained in experiment 8. During the stress period, the concentration of zinc-vitellogenin was markedly increased in non-supplemented birds compared to pre-stress values and the magnitude reached 63% ($P<0.01$). In vitamin E-supplemented birds, this increase was not observed and the concentration of zinc-vitellogenin was only very marginally increased compared to pre-stress values (+7%, N.S.).

Although the circulating concentration of zinc-vitellogenin was not measured in the present experiment, the increase in the liver concentration of zinc-vitellogenin in control birds was probably due to an accumulation of vitellogenin within hepatocytes in these birds during heat stress. Indeed previous studies (Utomo *et al.* (1994); experiment 8), demonstrated that, during heat stress, the concentration of circulating zinc-vitellogenin was decreased to a greater extent in control than in vitamin E-supplemented birds with decreases of 40% and 19% respectively. These results therefore suggested that, during the period of exposure to heat stress, the export of vitellogenin from the liver into the circulation was dramatically reduced in control birds but maintained in vitamin E-supplemented birds. The decreased export in control birds during heat stress resulted in a reduced concentration of the vitellogenin protein in the circulation and therefore lower availability of this protein for egg production.

This hypothesis could, however, not be confirmed by the observation of the subcellular localisation of the vitellogenin protein within hepatocytes by electron microscopy as the anti-vitellogenin antibody used in this experiment seemed to be non-specific. The specificity of this antibody was assessed in section 9.2.

9.2. Determination of the anti-vitellogenin antibody specificity

The specificity of the anti-vitellogenin antibody was assessed by the technique of immunoblotting (or western blotting).

9.2.1. Description of the technique

Immunoblotting is a technique used to identify specific antigens recognised by polyclonal or

monoclonal antibodies. Protein samples were solubilised to reduce disulphide bonds with sodium dodecyl sulphate (SDS). Following solubilisation, the material was separated by SDS-polyacrylamide gel electrophoresis or SDS-PAGE (section 9.2.2.). One-dimensional gel electrophoresis under denaturing conditions (i.e., in the presence of 0.1% SDS) separates proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode. The antigens were then electrophoretically transferred in a tank to a nitrocellulose membrane. The transferred proteins bound to the surface of the membrane, providing access for reaction with immunodetection reagents. All remaining binding sites were then blocked by immersing the membrane in a solution containing either a protein or detergent blocking agent. After probing with the primary antibody, the membrane was washed and the antibody-antigen complexes was identified with alkaline phosphatase enzymes coupled to the secondary anti-IgG antibody. The enzymes were attached via an avidin-biotin bridge to the secondary antibody and chromogenic substrates were used to visualise the activity.

9.2.2. Materials and methods

9.2.2.1. Preparation of the plasma sample

A sample of blood (5 ml) was collected from a mature laying hen, mixed with 100 µl of phenylmethylsulphonyl fluoride (PMSF) dissolved in isopropanol (70 mg of PMSF per ml of isopropanol) to inhibit proteolytic degradation of vitellogenin and placed on ice. Plasma was then collected (section 3.2.), analysed for protein concentration (section 3.2.1.) and stored at -20°C pending analysis.

9.2.2.2. SDS polyacrylamide gel electrophoresis

The polyacrylamide gel was a pre-cast gradient gel (size : 8 cm x 8 cm; thickness : 1 mm) containing 8 to 16% total acrylamide, kindly provided by Bob Scougall from the Roslin Institute and purchased from Novex (Encinitas, CA 92024). The gel was assembled as described below :

1. The cassette bag was opened. This was done by tearing the bag along an entire side starting from the notched corner. The buffer content was drained away and the cassette was removed by handling it by its edges only.
2. The tape covering the "slot" near the cassette's bottom was peeled off.

3. In one quick motion, the comb was pulled out of the cassette. This exposed the wells.
4. Using a Pasteur pipette, the cassette wells were washed with 1 x running buffer. The sample wells were left full of 1 x running buffer, without any air bubbles trapped.
5. The buffer core was oriented in the lower buffer chamber of the Xcell mini-cell gel box (electrophoresis tank) and the gel was inserted.
6. The rear wedge (with screw hole at top rear) was slided into the lower buffer chamber behind the front wedge.
7. The upper buffer chamber (corresponding to the void formed between the cassette and the buffer dam on each side of the buffer core) was filled with 1 x running buffer (approximately 150 ml).

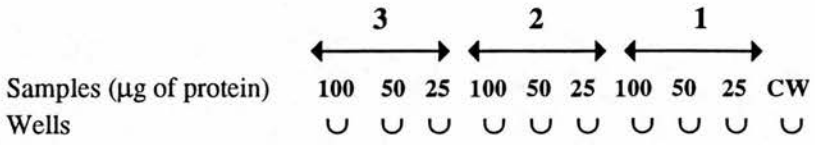
The samples were then prepared for loading onto the gel. An aliquot of 25 µl of the plasma sample prepared in section 9.2.2.1. was diluted to 600 µl with 575 µl of 2 x Laemmli sample buffer. As a protein concentration of 120 mg/ml was found in this plasma sample before dilution, the diluted solution contained 5 mg of protein per ml. This solution was boiled for 7.5 minutes to denature and reduce plasma proteins and then cooled. Different dilutions of this solution were then made using 2 x Laemmli buffer as described in Table 70.

Table 70 : Dilutions of the plasma protein sample.

Volume of the plasma protein solution (µl)	20	40	80
Volume of 2 x Laemmli buffer (µl)	60	40	0
Amount of protein contained in 20 µl of the solution (µg)	**25**	**50**	**100**

A protein-molecular-weight standards mixture, containing proteins of known molecular masses ranging between 29 and 205 kDa, was also loaded onto the gel as a control. 3 µl of this mixture was diluted with 7 µl of distilled H_2O and 10 µl of 2 x Laemmli buffer and the solution (20 µl) was loaded onto the gel. The samples and standard were then applied onto the gel using a 25 µl pipette equipped with round sample loading tips. 20 µl of protein samples were loaded into the wells by carefully applying the sample as a thin layer at the bottom of the wells. The control well (CW), first well on the right, was loaded with the molecular weight standards

mixture and the samples were loaded from right to left in the following order :



The lower buffer chamber was then filled by pouring approximately 500 ml of 1 x running buffer through the gap between the front gel and the front of the lower buffer chamber. The lid was placed on the buffer core. The power supply was connected to the cell and run at a constant voltage of 125 V until the bromo-phenol blue tracking dye had reached the bottom of the separating gel. This occurred within approximately 2 hours and the power supply was then disconnected. The lid and rear wedge and gel cassette were removed from the assembly. Each of the three bonded sides of the cassette were separated by inserting the knife's bevelled edge in the narrow gap between the cassette's two plates. The sharp edge of the gel knife was used to cut off the bottom lip of the gel. The gel was carefully removed from the lower plate, a small triangle was cut off one corner of the gel so the lane orientation was not lost during blotting and hybridisation.

9.2.2.3. Western blotting

After electrophoresis, the proteins were transferred from the gel onto a nitro-cellulose blotting membrane (Hybond) kindly provided by Caris Hogg from the Roslin Institute and purchased from Amersham International plc (Little Chalfont, U.K.). To perform this transfer, a sandwich containing the gel and the membrane was assembled as follows :

1. The blotting membrane was hydrated with distilled H_2O for 30 seconds. Then this membrane and two pieces of filter paper (Whatman 3MM) were soaked for 5 minutes in a dish containing 1 x transfer buffer. The gel was also soaked in this buffer for about 10 minutes.
2. A blotting pad was placed on the electrode of the negative electrode assembly.
3. A piece of filter paper was put on the pad and the surface was thoroughly wetted with 1 x transfer buffer.
4. The gel was picked up and placed on the filter paper, making sure that no air bubbles were trapped between the gel and the paper. The surface of the gel was wetted with about 1 ml

of 1 x transfer buffer and the blotting membrane was placed on the gel by starting at one edge and carefully laying the membrane down so that no bubbles were trapped between the membrane and the gel.

5. A wetted square of filter paper was placed on top of the membrane making sure that no bubbles were trapped between the paper and the membrane. Two Scotchbrite pads were placed on top of the filter paper.

6. The positive electrode assembly was placed on top of the gel/Scotchbrite stack and the entire assembly was pressed together. The assembly was then placed in the tank so that the plus sign on the positive electrode was seen from the front of the tank. While holding the assembly together at the top, the front wedge was placed so that its vertical face was against the assembly. The rear wedge was slid in and pushed down firmly.

7. About 50 ml of 1 x transfer buffer was poured into the assembly and it was checked the latter was properly sealed. Then more 1 x transfer buffer was poured on the assembly until a small amount of buffer spilled over the gasket into the tank.

8. The tank around the assembly was filled with water to about 2 cm from the top of the tank.

9. The lid was placed on the assembly, the leads were connected to the power supply and the power supply turned on at a constant voltage of 30 V for 2 hours.

10. Once the transfer was terminated, the sandwich was disassembled, the membrane was collected and rinsed in water.

9.2.2.4. Hybridisation

Once rinsed, the membrane was cut in three to isolate the three replicates of samples and the first replicate was left attached to the molecular weight standards mixture. The three pieces of membrane were then placed in a hydridisation bottle and the following procedure was followed :

1. 10 ml of Marvel solution and 50 µl of normal goat serum (Vectastain alkaline phosphatase Rabbit IgG kit purchased from Vector Laboratories, Burlingame, CA 94010) were added into the bottle. The bottle was then closed making sure no solution leaked and placed on a Spira-mix at 4°C overnight. This was done to block the sites of the membrane which have no protein.

2. The next morning, the two pieces of the membrane corresponding to the first and second

replicates of sample were removed from the bottle. The piece corresponding to the first replicate of samples was placed in a second hybridisation bottle containing 10 ml of Marvel solution, 50 µl of normal goat serum and 7.5 µl of chicken anti-vitellogenin antibody raised in rabbit (corresponding to a dilution of **1:1333** for the antibody). The piece of the membrane corresponding to the second replicate of samples was placed in a third hybridisation bottle containing 10 ml of Marvel solution, 50 µl of normal goat serum and 15 µl of chicken anti-vitellogenin antibody (corresponding to a dilution of **1:667** for the antibody). The piece of the membrane corresponding to the third replicate of samples was left in the first hybridisation bottle with the same solution as was used overnight. These bottles were placed on a Spira-mix at room temperature for 3 hours.

3. The three bottles were then emptied and the membranes were rinsed three times with TTBS for 5 minutes each time.

4. The bottles were emptied and the three pieces of membrane were put in the same first hybridisation bottle in which 10 ml of Marvel solution, 50 µl of normal goat serum and 50 µl of biotinylated, affinity-purified anti-rabbit IgG (Vectastain alkaline phosphatase Rabbit IgG kit purchased from Vector Laboratories, Burlingame, CA 94010). The bottle was then placed on a Spira-mix at room temperature for 1.5 hours.

5. The bottle was emptied and the membranes were rinsed three times with TTBS for 5 minutes each time.

6. The bottle was emptied and 10 ml of TTBS, 100 µl of avidin DH (reagent A from Vectastain ABC-AP kit purchased from Vector Laboratories, Burlingame, CA 94010) and 100 µl of biotinylated alkaline phosphatase H (reagent B from Vectastain ABC-AP kit) were added into it. The bottle was then placed on a Spira-mix at room temperature for 30 minutes to allow complex formation.

7. The bottle was emptied and the membranes were rinsed three times with TTBS for 5 minutes each time.

8. The bottle was emptied and 5 ml of substrate solution were added to it. The bottle was then placed on a Spira-mix at room temperature until suitable staining developed (approximately 1 minute).

9. The bottle was emptied and the membranes were washed in distilled water for 5 minutes.

9.2.2.5. Solutions

1 x Running buffer

2.9 g of Trisbase, 14.4 g of glycine and 1 g of SDS were mixed in 1 litre of distilled H_2O. The pH was approximately 8.3.

2 x Laemmli Buffer

10 ml of 0.5 M Tris/HCl pH 6.8 were added to 10 ml of 10% SDS and 10 ml of glycerol. A small amount of bromo-phenol blue was also added to this solution and the volume was made up to 100 ml with distilled H_2O.

1 x Transfer buffer

1.45 g of Tris, 7.21 g of glycine were mixed in 800 m1 of distilled H2O. The pH was approximately 8.3. 200 ml of methanol was then added to this solution making up a total volume of 1 litre.

Marvel solution

0.4 g of dried skimmed milk was mixed to 10 ml of TTBS.

TTBS

12.1 g of Tris and 9 g of NaCl were mixed in 1 litre of distilled H_2O. pH was adjusted to 7.5 with 5 N HCl and 1 ml of polyoxyethylenesorbitan monolaurate (Tween 20) was added.

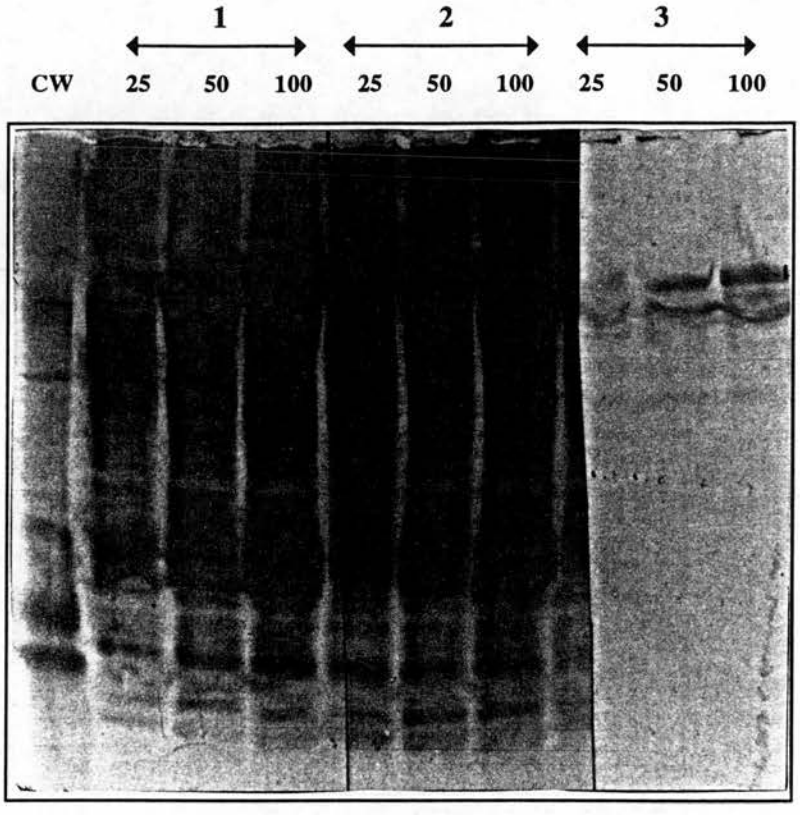
Substrate solution

0.121 g of Tris/HCl was mixed to 10 ml of water. The pH was approximately 8.2. Three reagents (reagents 1, 2 and 3 from the Vectastain alkaline phosphatase Rabbit IgG kit purchased from Vector Laboratories, Burlingame, CA 94010) were then added to this solution. 200 µl of reagents 1, 2 and 3 were added sequentially to this solution with mixing after addition of each reagent. The solution was used immediately.

9.2.3. Results

The nitro-cellulose membrane was photographed following hybridisation and staining and is presented below (Figure 31).

Figure 31 : Western blot of laying hen plasma proteins with anti-vitellogenin antibody (replicate 1 : antibody dilution of 1:1333; replicate 2 : antibody dilution of 1:667 and replicate 3 : no antibody).

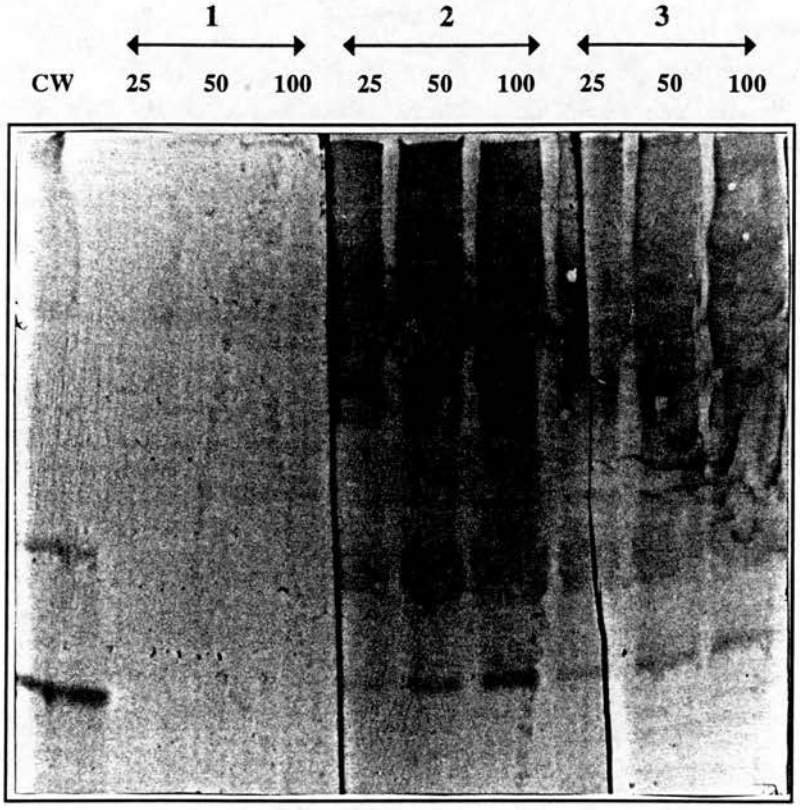


There was significant smearing in all the lanes of replicates 1 and 2 despite the fact that different amounts of protein were analysed. This could not have been due to the non-specific binding of the second antibody as no smearing occurred for the replicate 3. However, this could have been due to a high titre of the antibody (the actual titre of the antibody could not be provided by the source laboratory and was unknown). To rule out this possibility, the experiment was repeated using a higher dilution, 1:10,000 for the second replicate of samples and 1:20,000 for the third replicate of samples. These corresponded to a further dilution of 15 times over that used in the first experiment. The first replicate was not treated with the primary antibody and used as a control to detect non-specific binding of the secondary antibody. The samples were loaded as follows :

	3			2			1			
Samples (µg of protein)	100	50	25	100	50	25	100	50	25	CW
Wells	∪	∪	∪	∪	∪	∪	∪	∪	∪	∪

The nitro-cellulose membrane was photographed following hybridisation and staining and is presented below (Figure 32).

Figure 32 : Western blot of laying hen plasma proteins with anti-vitellogenin antibody (replicate 1 : no antibody; replicate 2 : antibody dilution of 1:10,000 and replicate 3 : antibody dilution of 1:20,000).



This photograph revealed that, despite the massive dilutions of the anti-vitellogenin antibody, significant smearing appeared in all the lanes of replicates 2 and 3.

9.2.4. Discussion

The determination of the anti-vitellogenin antibody specificity demonstrated that this antibody was not specific to vitellogenin and was of doubtful quality. The gold labelling found in the sections observed by immunoelectron microscopy could therefore not be attributed to vitellogenin and may explain why gold particles were found in organelles which do not contain vitellogenin.

9.3. Conclusion

The first objective of experiment 9 was to determine whether the results obtained in experiment 8, and in particular the effects of heat stress and vitamin E on the liver concentration of vitellogenin protein, could be reproduced. As the results of experiment 8 were obtained on small numbers of birds, experiment 9 was carried out on a larger number of birds. The minimal number of replications required to have an 80% chance of obtaining a significant difference between the control and vitamin E-supplemented groups was determined and this calculation demonstrated that, in order to obtain a significant difference between these two groups, a minimum of 10 birds per group should be used. This was done during the stress period and an additional 3 birds per group were also used during the thermoneutral period.

The liver vitellogenin concentration detected in these birds confirmed the observations made in experiment 8. During the thermoneutral period, the concentration of vitellogenin was similar in both control and supplemented birds and found to be approximately 2.34 ± 0.88 μg per g of liver (versus 2.15 ± 0.42 μg per g of liver in experiment 8). During the period of stress, the concentration of vitellogenin was markedly increased in the control group and reached 3.81 ± 0.95 μg per g of liver (versus 3.80 ± 0.86 μg per g of liver in experiment 8). In the vitamin E-supplemented group, such an increase did not occur and the concentration of vitellogenin in the liver was maintained to a closer value as before the stress and was 2.50 ± 1.07 μg per g of liver (versus 2.38 ± 0.95 μg per g of liver in experiment 8).

Taken together, experiments 8 and 9 therefore demonstrated that the concentration of vitellogenin in the liver was significantly higher in control than in vitamin E-supplemented birds (+52%, $P<0.01$) during heat stress. This suggested that, during heat stress, the export of vitellogenin from the liver to the circulation was impaired in laying hens fed a control diet (10 mg vitamin E per kg diet). This could explain the reduced concentration and therefore availability of vitellogenin in the circulation and the reduced egg production. In birds fed a vitamin E-supplemented diet (300 mg vitamin E per kg diet), the accumulation of vitellogenin in their liver did not occur, a higher concentration of vitellogenin could therefore be observed in the circulation and this could explain the improved egg production observed during heat stress. The observations of liver sections by immunoelectron microscopy, performed to determine the subcellular localisation of vitellogenin within hepatocytes, could unfortunately not confirm this hypothesis as the anti-vitellogenin antibody used in this experiment was probably not specific for vitellogenin.

Chapter 10

General discussion

The initial objective of the research was to test the hypothesis that dietary vitamin E supplementation could alleviate detrimental effects of heat stress on egg production in laying hens. This hypothesis was tested under field conditions in two large scale experiments (Chapter 4). **Experiment 1** showed that, in birds exposed to a period of chronic heat stress (4 weeks at 32°C) between 24 and 28 weeks of age and fed a vitamin E-enriched diet (500 mg vitamin E /kg diet) from 20 to 40 weeks of age, egg production was significantly improved during the period of stress (+9.2%, $P<0.05$) compared to birds fed a control diet (10 mg vitamin E per kg diet). The superior production was also found to extend beyond the period of stress, as supplemented birds showed superior egg production during the two months following heat stress (+10.5%, $P<0.01$) compared to controls. **Experiment 2**, designed to determine the most effective vitamin E regimen, demonstrated that a lower dose of vitamin E (250 mg per kg diet) provided for four weeks before, during and after heat stress maximised egg production under stressful conditions. Increases of 11.7% and 12.6% ($P<0.02$) respectively were obtained during heat stress and in the four weeks following. These experiments thus confirmed the hypothesis and established the most effective dietary vitamin E concentration for maximising egg production under chronic heat stress.

Subsequent research was aimed at understanding the biochemical mechanism(s) by which vitamin E improved egg production in heat stressed laying hens. As mentioned in the literature review (Chapter 2), a preliminary study by Utomo *et al.* (1994) demonstrated that, concurrent with the decrease in egg production, heat stress resulted in a decrease in the circulating concentration of yolk precursors and that the improvement in egg production resulting from dietary vitamin E supplementation (500 mg vitamin E per kg diet) was associated with an elevation in yolk precursor concentration. The effects of heat stress and vitamin E on the main steps of egg formation were investigated in order to understand how these factors could influence the concentration of yolk precursors. The process of egg formation is strictly

controlled by the endocrine system, particularly, by the reproductive hormones. These hormones include the oestrogens which are produced by the growing oocytes in the ovary. Oestrogens are involved in a wide range of functions ranging from the development of secondary sexual characteristics and initiation of sexual behaviour to the synthesis of yolk precursors by the hepatocytes. The latter, which occurs exclusively in the liver, is an important step in the process of egg production as the synthesis of yolk precursors is a prerequisite for yolk formation. Once yolk precursors are synthesised, they are exported from the liver cells, transported via the circulation towards the ovary and are actively taken up by oocytes. Therefore, egg formation involves the mutually dependent activities of the ovary and of the liver. To determine how vitamin E improves egg production in heat-stressed birds, the influences of chronic heat stress and vitamin E on these two organs were investigated. The effects of heat stress and vitamin E on the uptake of yolk precursors by oocytes, the circulating concentration of oestrogens, the synthesis of yolk precursor by hepatocytes and the export of yolk precursors from the hepatocytes into the circulation were studied.

To measure the uptake of yolk precursors by oocytes, a technique which involved preparing a pure solution of radiolabelled vitellogenin, the main yolk precursor protein, was developed (Chapter 4). This solution was then injected into mature laying hens in **experiment 3,** and after given periods of time, the birds were killed and samples of blood, pectoral muscle, liver and oocytes were collected. Radioactivity was successfully detectable in blood, liver and oocytes. This experiment demonstrated that, in order to relate the radioactivity found in the oocytes to the uptake of vitellogenin, the concentration of the total vitellogenin in the circulation had to be determined prior to the injection of ^{65}Zn-labelled vitellogenin. This procedure was adopted in the two following experiments in which the effects of chronic heat stress and vitamin E on the uptake of vitellogenin by oocytes *in vivo* were studied.

In **experiment 4**, the uptake of vitellogenin by oocytes was found to be reduced by 52% in birds exposed to heat stress compared to birds housed in thermoneutral conditions. The magnitude of the decrease was similar to the magnitude of the decrease in the circulating vitellogenin concentration (57%). This suggested that, rather than being directly affected by heat stress, the uptake of vitellogenin was dependent on the amount of vitellogenin available in the circulation. However, as this result did not exclude a possible effect of vitamin E on the uptake of vitellogenin by oocytes, the latter was also measured in **experiment 5** in birds fed a control diet (10 mg vitamin E per kg diet) or a vitamin E-supplemented diet (250 mg vitamin E per kg diet) and exposed to a chronic period of heat. This experiment demonstrated that the

uptake of vitellogenin by oocytes was similar in both groups of birds. As the concentration of vitellogenin in the circulation was also found to be similar in both groups of birds, experiments 4 and 5 therefore confirmed that the uptake of vitellogenin by oocytes was dependent on the amount of vitellogenin available in the circulation.

The objective of the remaining experiments was to establish the mechanism(s) involved in the regulation of plasma vitellogenin. A possible determinant of the concentration of yolk precursors in the circulation is the degree of activity of the genes encoding for these precursors. As this activity is, to a large extent, controlled by the concentration of oestrogens in the circulation, the circulating concentration of 17β-oestradiol in birds exposed to thermoneutral or heat stress conditions and fed control or vitamin E-supplemented diets was investigated in Chapter 7.

To determine the best RIA method for measuring the concentration of 17β-oestradiol, two techniques for purification and two techniques of quantitation were tested in **experiment 6** using samples of immature laying hen plasma spiked with different amounts of 17β-oestradiol. The two methods of purification that were tested were extraction by solvent and extraction by affinity chromatography and samples were quantitated either as described in the Pantex kit or as described by Webb *et al.* (1985). As more consistent results were obtained in the samples extracted by affinity chromatography and quantitated by the method of Webb *et al.* (1985), the combination of these two techniques was therefore employed in **experiment 7**. The aim of this experiment was to determine the concentration of 17β-oestradiol in the circulation of birds exposed to thermoneutral or heat stressed conditions and fed a control diet (10 mg vitamin E per kg diet) or a vitamin E-supplemented diet (250 mg vitamin E per kg diet). Contrary to expectations, neither heat stress nor vitamin E were found to have any effect on the circulating concentration of 17β-oestradiol. The changes, if any, in the stimulation of the genes encoding for yolk precursors are therefore not likely to be due to a direct effect of heat stress or of vitamin E on the concentration of oestrogen hormones.

Experiments were then carried out to determine whether heat stress or vitamin E had any effect on liver vitellogenin production. **Experiment 8** was designed to determine whether these factors influenced the amount of vitellogenin mRNA and the concentration of vitellogenin in the liver. The amount of vitellogenin transcript was quantitated by Northern Blot Analysis in birds exposed to thermoneutral or heat stress conditions and fed a control diet (10 mg vitamin E per kg diet) or a vitamin E-supplemented diet (300 mg vitamin E per kg diet). This experiment

demonstrated that, during heat stress, the amount of vitellogenin transcript was significantly reduced (-60%, $P<0.01$) in both control and vitamin E-supplemented birds. This experiment also demonstrated that the concentration of the vitellogenin protein in the circulation was significantly reduced during heat stress in birds fed the control diet, but less so in birds fed the vitamin E-supplemented diet, an observation which was also made by Utomo *et al.* (1994). The determination of the concentration of vitellogenin in the liver revealed more surprising results. Contrary to expectations, the liver concentration of vitellogenin was significantly increased during heat stress in control birds (+77%, $P<0.01$) compared to pre-stress values. Such an increase was not observed in the birds fed the vitamin E-supplemented diet and the liver concentration of vitellogenin in these birds was significantly lower than in the control birds (-60%, $P<0.05$). The ratio of concentration of vitellogenin in the circulation to concentration of vitellogenin in the liver was therefore decreased in control birds and the magnitude of the decrease was found to be greater in the birds showing the lower egg production. Depressed egg production therefore seemed to be correlated with high liver and low circulating concentrations of vitellogenin.

This observation provided a potential explanation for the effects of heat stress and vitamin E on egg production, but since experiment 8 was only carried out on a small number of birds (5 birds in each control and supplemented group), another experiment was performed to determine whether this result could be reproduced on a larger scale. The minimal number of replications required to have 80% chance of obtaining a significant response was calculated as described in Chapter 9 and was found to be 10 birds per group. Therefore, in **experiment 9**, the effects of heat stress and vitamin E were tested on 20 birds. The results obtained in this experiment confirmed that the liver concentration of vitellogenin was significantly increased during heat stress in birds fed a control diet (10 mg vitamin E per kg diet) compared to pre-stress values. This experiment also confirmed that this effect was not observed in vitamin E-supplemented birds (300 mg vitamin E per kg diet). These observations suggested that the export of vitellogenin from the liver into the circulation was reduced in heat stressed birds fed a control diet (10 mg vitamin E per kg diet) with the resulting accumulation of vitellogenin protein.

Accumulation of vitellogenin protein in hepatocytes of control birds during heat stress could be due to impaired secretion of the protein from the hepatocytes into the circulation. To test this hypothesis, an attempt was made to visualise the subcellular localisation of the vitellogenin protein by the technique of immunoelectron microscopy using liver sections obtained from control and vitamin E-supplemented birds in thermoneutral and heat stress conditions. The

technique relies on the use of an anti-chicken vitellogenin antibody which was obtained from D. Williams at the State University of New York. The results of this experiment were however not very conclusive. The hybridisation signals were very low and gold particles, indicating where the antibody had hybridised, were found in organelles which should not contain vitellogenin. To test the specificity of the antibody, a western blot was performed on plasma protein samples and revealed that this particular antibody preparation was not specific for vitellogenin.

Taken together, the results of experiments 8 and 9 appear to support the hypothesis that vitamin E improves egg production during heat stress by facilitating a greater export of vitellogenin from the liver to the circulation and a consequent greater availability of yolk precursors in the circulation. This effect is probably related to the antioxidant properties of vitamin E. As reviewed in Chapter 2, exposure to stress is known to result in an increased production of free radicals and ROS. These, in turn, are known to cause damage to a wide range of molecules but, particularly, to lipids by initiating a chain peroxidation reaction. By scavenging these free radicals and ROS, antioxidants such as vitamin E reduces this effect. Vitamin E is mainly stored within cellular and organelle membranes and these structures may be protected to a greater extent against lipid peroxidation in vitamin E-supplemented than in control birds. Membrane integrity may be required for proper functioning of the secretory pathway which is the main route by which vitellogenin is exported outside the hepatocytes (Kami and Stoward, 1985). It is thus proposed that heat stress depresses egg production by impairing the transmembrane transport of vitellogenin from the liver and that vitamin E alleviates this effect of heat stress by promoting better membrane stability and maintaining more normal transfer of vitellogenin from liver into the circulation.

However, and to be more comprehensive, the study of the effects of vitamin E in heat-stressed birds would benefit from further analysis of the influence of vitamin E on food intake. Indeed, although no significant effect of vitamin E was demonstrated on food intake, it seems that vitamin E-supplemented birds showed a slightly greater food intake than the non-supplemented birds. It is therefore possible that vitamin E, by some direct or indirect mechanisms, increases food consumption and thus egg production. Pair-feeding experiments could be carried out to study the effect of vitamin E on food intake in heat-stressed birds. In particular, the effect of vitamin E on some of the factors regulating food intake (e.g. leptin) or on the intensity of the respiratory disorders (e.g. ascites) occuring during heat stress should be investigated.

To provide evidence for this hypothesis and to understand the exact mechanisms by which the export of yolk precursors is affected during heat stress, further experiments could be undertaken. The respective degrees of involvement of heat stress and reduction of food intake on the accumulation of vitellogenin in hepatocytes could be studied by determining the liver concentration of vitellogenin in two groups of birds fed a control diet : birds exposed to heat stress or birds housed in thermoneutral conditions but fed a reduced amount of food (pair-feeding experiment). The concentration of vitellogenin in the liver of heat stressed and vitamin E-supplemented birds could also be determined by using an independent technique from the one described in this thesis. Samples of liver could, for example, be collected from the different groups of birds, processed and analysed for their vitellogenin concentration by western blotting. This, of course, requires the availability of a specific antibody raised against chicken vitellogenin. If a specific antibody is obtained, another attempt to localise the vitellogenin protein in the liver by immunoelectron microscopy could also be undertaken.

Annexe

Table A1 : Composition and characteristics of the pre-lay diets (Experiment 1).

	From 16 to 18 weeks of age	From 19 to 20 weeks of age
Composition (%)		
Yellow corn	47.45	51.5
Bran	13.00	3.60
Field pea	10.00	7.00
Meat and bone meal (50%)	5.90	7.00
Alfalfa	6.00	1.90
Wheat flour	5.00	0.00
Soybean meal (48%)	5.20	13.60
Rapeseed	3.00	2.00
Rapeseed meal	1.70	0.00
Sunflower meal	0.70	1.90
Extruded soybean	0.00	2.00
Fat	0.30	0.00
Rapeseed oil	0.00	1.00
Methionine adjuvant (15%)	0.15	0.40
Limestone	0.60	7.10
Vitamins & minerals	1.00	1.00
Characteristics		
Metabolisable energy (kcal/kg)	2,730	2,750
Crude proteins (%)	15.5	15.5
Lysine %	0.77	0.90
Methionine (%)	0.30	0.36
Methionine + cystine (%)	0.59	0.67
Tryptophan (%)	0.15	0.17
Calcium (%)	1.20	3.80
Available phosphorus (%)	0.35	0.33

Table A2 : Composition and characteristics of the basal diet (Experiment 1).

Composition (%)		**Characteristics**	
Yellow corn	61.10	Metabolisable energy (kcal/kg)	2,850
Field pea	11.00	Crude proteins (%)	15.5
Soybean meal (48%)	10.00	Lysine %	0.77
Meat and bone meal (50%)	5.00	Methionine (%)	0.33
Alfalfa meal (17%)	2.00	Methionine + cystine (%)	0.60
Corn oil	1.50	Calcium (%)	3.50
Limestone	3.40	Available phosphorus (%)	0.30
Minerals (*)	5.00		
Premix (**)	0.30		

(*) Supplies per kg of minerals : Limestone 815; Dicalcium phosphate 81; Salt 60; Choline (50%) 30; Micronutrients 10; Vitamins (without vitamin E) 5.

(**) Supplies per kg of premix : DL Methionine 100; Microvit E 50 ADS 2 (diet containing 10 mg Vit.E/kg), 15 (diet containing 125 mg Vit.E/kg) or 63 (diet containing 500 mg Vit.E/kg), Complement of ground maize 898, 885 or 837.

Table A3 : **Composition and characteristics of the pre-lay diets (Experiment 2).**

	From 16 to 19 weeks of age	From 19 to 22 weeks of age
Composition (%)		
Yellow corn	47.45	51.5
Bran	13.00	3.60
Field pea	10.00	7.00
Meat and bone meal (50%)	5.90	7.00
Alfalfa	6.00	1.90
Wheat flour	5.00	0.00
Soybean meal (48%)	5.20	13.60
Rapeseed	3.00	2.00
Rapeseed meal	1.70	0.00
Sunflower meal	0.70	1.90
Extruded soybean	0.00	2.00
Fat	0.30	0.00
Rapeseed oil	0.00	1.00
Methionine adjuvant (15%)	0.15	0.40
Limestone	0.60	7.10
Vitamins & minerals	1.00	1.00
Characteristics		
Metabolisable energy (kcal/kg)	2,730	2,750
Crude proteins (%)	15.5	15.5
Lysine %	0.77	0.90
Methionine (%)	0.30	0.36
Methionine + cystine (%)	0.59	0.67
Tryptophan (%)	0.15	0.17
Calcium (%)	1.20	3.80
Available phosphorus (%)	0.35	0.33

Table A4 : Composition and characteristics of the basal diet (Experiment 2).

Composition (%)		**Characteristics**	
Yellow corn	61.60	Metabolisable energy (kcal/kg)	2,840
Soybean meal (48%)	10.80	Crude proteins (%)	14.8
Field pea	8.00	Lysine %	0.89
Meat and bone meal (50%)	5.00	Methionine (%)	0.43
Soyabean oil	1.90	Methionine + cystine (%)	0.65
Salt	0.30	Calcium (%)	4.40
Limestone	10.00	Available phosphorus (%)	0.57
Dicalcium phosphate	0.40		
Minerals (*)	1.00		
Premix (**)	1.00		

(*) Supplies per kg of minerals : Choline (50%) 150; Micronutrients 50; Vitamins (without vitamin E) 25; Starch 775.

(**) Supplies per kg of premix : DL Methionine 230; L Lysine 220; Microvit E 50 ADS 0 (diet containing 10 mg Vit.E/kg), 23 (diet containing 125 mg Vit.E/kg), 48 (diet containing 250 mg Vit.E/kg), 73 (diet containing 375 mg Vit.E/kg) or 98 (diet containing 500 mg Vit.E/kg), Complement of ground maize 550, 527, 502, 477 or 452.

Abbreviations

Ab : antibody
ACTH : adrenocorticotrophic hormone
Ag : antigen
apo-B : apolipoprotein-B
ATP : adenosine triphosphate
AVT : arginine vasoticin
CBG : corticosteroid-binding globulin
cDNA : complementary deoxyribonucleic acid
CP : crude protein
CPM : counts per minute
DEPC : diethyl pyrocarbonate
DNA : deoxyribonucleic acid
E_2 : estradiol (oestradiol)
EDTA : ethylene diamine tetra acetate
ER : endoplasmic reticulum
FCE : food conversion efficiency
FSH : follicle stimulating hormone
GH : growth hormone
GnRH : gonadotrophin releasing hormone
GSH : reduced glutathione
HDL : high density lipoprotein
HIV : human immunodeficiency virus
HPLC : high pressure liquid chromatography
HSE : heat shock element
HSF : heat shock factor
HSP : heat shock protein
IgG : immunoglobulin G
ISV : immature secretory vacuole

kcal : kilocalorie

kDa : kilodalton

LDL : low density lipoprotein

LH : luteinising hormone

LHRH : luteinising hormone releasing hormone

LSD : long supplementation duration

MDA : malondialdehyde

ME : metabolisable energy

mRNA : messenger ribonucleic acid

MSV : mature secretory vacuole

MT : mesotocin

NAD : nicotinamide adenine dinucleotide

NADH : reduced nicotinamide adenine dinucleotide

NADPH : reduced nicotinamide adenine dinucleotide phosphate

NS : non significant

NSB : non specific binding

OR : oestrogen receptor

ORE : oestrogen response element

PBS : phosphate buffered saline

PHS : pulmonary hypertension syndrome

PMSF : phenylmethylsulphonyl fluoride

PUFA : poly unsaturated fatty acid

RES : reticulo-endothelial system

RER : rough endoplasmic reticulum

RH : relative humidity

RNA : ribonucleic acid

rRNA : ribosomal ribonucleic acid

RT : room temperature

RIA : radioimmunoassay

ROS : reactive oxygen species

SDS : sudden death syndrome (Chapter 2)

SDS : sodium dodecyl sulphate (Chapter 9)

SED : standard error of difference of means

SHBG : sex-hormone-binding globulin

SOD : superoxide dismutase

SSD : short supplementation duration

TBA : thiobarbituric acid

TeBG : testosterone-binding globulin

UV : ultraviolet

Vg : vitellogenin

Vg-Zn : vitellogenin-bound zinc

VLDL : very low-density lipoprotein

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CARFAX

Influence of high dietary vitamin E supplementation on egg production and plasma characteristics in hens subjected to heat stress

S. BOLLENGIER-LEE[1], M. A. MITCHELL, D. B. UTOMO, P. E. V. WILLIAMS[1] AND C. C. WHITEHEAD

Roslin Institute (Edinburgh), Roslin, Scotland and [1]Rhône-Poulenc Animal Nutrition, Antony, France

Abstract 1. The effects of different dietary concentrations of vitamin E (α-tocopherol acetate) were investigated in 2 experiments on laying hens exposed to chronic heat stress at 32°C.

2. In the first experiment, egg production and plasma concentrations of calcium and egg yolk precursors were measured in 24 hens before, during and after a stress period of one week and fed on diets containing 10 or 500 mg vitamin E/kg.

3. In the second, larger experiment, egg production and food intake were measured in 300 hens housed in 2 temperature-controlled rooms and fed on diets containing 10, 125 or 500 mg vitamin E/kg. Birds in room 1 were stressed from 24 to 28 weeks of age and those in room 2 from 32 to 36 weeks.

4. In experiment 1, egg production and egg weight were significantly higher (72·6 *vs* 51·2%, $P < 0·05$ and 66·6 *vs* 63·1 g, $P < 0·005$ respectively) during and after the period of stress in the group given 500 mg vitamin E/kg. Plasma concentrations of calcium, vitellogenin (zinc) and VLDL (triglyceride) were also higher in this group.

5. In experiment 2, egg production was significantly higher (65·4 *vs* 56·2%, $P < 0·05$) during and after the period of heat stress in birds in room 1 fed on the diet containing 500 mg vitamin E/kg. Egg production was also higher (49·9% *vs* 44·7%) on this treatment during the stress period in room 2, though the difference was not significant ($P < 0·10$). Egg weight and food intake were unaffected by treatment in either room.

6. It is concluded that dietary supplementation with extra vitamin E can, at least in part, alleviate the adverse effects of chronic heat stress in laying hens, perhaps by maintaining the supply of egg precursors in plasma.

INTRODUCTION

Stress is a general subjective term used to describe the sum of non-specific responses or defence mechanisms of the body when faced with abnormal or extreme demands. Many factors, including environmental, nutritional and pathological disturbances, can generate a state of stress and evoke a combination of behavioural, biochemical and physiological adaptations which generally result in a reduction in the performance of poultry. Heat stress in particular has a highly detrimental effect on egg production in the laying hen; both the number and weight of eggs are markedly decreased when the bird is exposed to high ambient temperatures (Smith, 1974). The plasma concentrations of very low density lipoprotein (VLDL) and vitellogenin, which are egg yolk precursors, are also reduced during heat stress (Utomo *et al.*, 1994). Depressed eggshell thickness was also observed by Ahvar *et al.* (1981) resulting in a poorer egg shell quality and reduced shelf-life.

Considerable attention has been paid to the role of nutrition in minimising the effects of heat stress (Austic, 1985; Leeson, 1986; Shane, 1988). Exposure of heat-stressed laying hens to periods of starvation has been shown to increase egg production (Huthail, 1992). Feeding laying hens a separate protein, energy and often calcium source has also been shown to be beneficial (Farrell *et al.*, 1981). Manipulations of the dietary energy density or protein content have been studied but gave conflicting results (Leeson, 1993; Ramlah and Sarinah, 1992). Provision of mineral-enriched water to layers has improved shell quality (Odom *et al.*, 1985). Vitamin C was reported to improve the performance of poultry in a hot environment. It increased egg production, improved hatchability and fertility and reduced egg breakage and mortality (Thornton and Moreng, 1959).

It is well established that heat stress can lead to a reduction in the birds' defence mechanisms or to a relative state of immunosuppression (Thaxton

Correspondence to: Dr C. C. Whitehead, Roslin Institute (Edinburgh), Roslin, Midlothian EH25 9PB, Scotland. Email: Colin.Whitehead@bbsrc.ac.uk.

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and Siegel, 1970). It is also accepted that stress can lead to over production of oxygen free radicals OH· and O_2^- (Slater, 1984). Free radicals can cause metabolic disturbances and cell injury in many ways. A reactive free radical formed close to DNA may produce a change in the molecular structure resulting in a mutation or cytotoxicity (Collins *et al.*, 1994) and cause profound changes in enzyme activity. Reactive free radicals may also damage cells by lipid peroxidation of polyunsaturated fatty acids (PUFAs). This is, by far, the most important damage produced by free radicals in the animal (Halliwell and Gutteridge, 1989). Increased oxidative stress and liver lipid peroxidation were observed in chickens fed on a diet rich in PUFAs and the condition was made worse when the birds were fed on a vitamin E-deficient diet (Fuhrmann and Sallmann, 1995).

Vitamin E, through its intra-membrane antioxidant properties (Tappel, 1972), may protect tissue membranes from lipid peroxidation caused by free radical attack. It could, therefore, reduce the associated loss of integrity of function of cell membranes and associated increased cellular permeability and play a role in alleviating the effect of heat stress in laying hens. To investigate the possible role of vitamin E in reducing some of the effects of heat stress 2 experiments were carried out. The objective of the first experiment was to determine whether a high dose of vitamin E (500 mg of DL-α-tocopherol acetate/kg of diet) had any positive effect on laying hens exposed to a chronic heat stress and to characterise possible mechanisms mediating such actions, including the influence on plasma egg yolk precursors. The objective of the second experiment was to investigate, on a larger scale, the effect of high dietary doses of vitamin E (125 and 500 mg DL-α-tocopherol acetate/kg) on egg laying hens exposed to chronic heat stress.

MATERIALS AND METHODS

Experiment 1

Diet and husbandry

Twenty four ISA Brown birds, aged between 35 and 38 weeks, were housed in individual cages (46 × 29 × 42 cm) in a climate room at 22°C. After one week of adaptation, 24 hens were distributed at random into 2 groups and fed on either a control diet (10 mg of DL-α-tocopherol acetate/kg) or the same diet supplemented with 500 mg DL-α-tocopherol acetate/kg. The basal diet was a standard layer mash containing 160 g CP, 11 MJ ME and 3 g calcium/kg. The lighting schedule was 14h light: 10h dark. After 2 weeks of acclimatisation, the temperature was increased to 32°C for one week and then returned to 22°C. The relative humidity was actively controlled and maintained constant at 35%. Egg number and egg weight were measured daily. Blood samples (2 ml) were taken from the brachial vein of each bird twice before the heat stress (on days 1 and 13), 3 times during the heat stress (days 16, 19 and 22) and twice after the heat stress (days 26 and 29). These samples were then transferred to heparinised tubes and placed on ice. Plasma samples were prepared by centrifugation of blood at 1,500 *g* for 5 min, frozen immediately and stored at −20°C until analysed. Plasma concentrations of 3 variables involved in egg production, calcium ions, vitellogenin and VLDL, were determined. Plasma zinc ion concentration was measured as an indicator of vitellogenin (Mitchell and Carlisle, 1991) and triglyceride concentration as an indicator of VLDL.

Analyses

Plasma total calcium concentration was measured using a commercial kit (Calcium C-Wako Chemicals GmbH) modified for use with avian body fluids and for absorbance determination on an automatic plate reader (Titertek Twinreader® PLUS, Flow Laboratories, UK). Plasma zinc and triglyceride concentrations were determined using commercial kits (Zn-Wako Chemicals GmbH and Triglyceride-N, Wako Chemicals GmbH). All Wako products were supplied by Alpha Laboratories, UK.

Individual bird data were analysed within groups and were compared on a weekly basis using 2-way analysis of variance (Anova) and *t* test (Minitab statistical package).

Experiment 2

Diet and husbandry

Six hundred ISA Brown hens were housed at 16 weeks of age in individual cages in a climate room at 22°C for an adaptation period of 4 weeks. Between 16 and 18 weeks, the pullets were fed on a pelleted diet containing 155 g CP, 11·4 MJ ME and 12 g calcium/kg, and between 18 and 20 weeks of age, they were fed on a diet containing 155 g CP, 11·5 MJ ME and 38 g calcium/kg. At 16 and 20 weeks of age, the birds were individually weighed, the live-weight gain between weeks 16 and 20, the body weight and the pelvic gap at week 20 were used as criteria to select 300 birds in good laying condition. These birds were then housed in individual cages in 3 tier battery units in 2 temperature controlled rooms. The light period was initially 9 h per day but was progressively increased by one hour week to reach 15:5 hr in week 24. Between 20 and 40 weeks of age, the birds were fed on either the control diet (10 mg DL-α-tocopherol acetate/kg) or an identical diet but supplemented with 125 or 500 mg of DL-α-tocopherol

acetate kg. These diets were allocated according to a blocked design in each room. The basal diet was a pelleted layer diet (pellets of 2·5 mm of diameter) containing 155 g CP, 11·9 MJ ME and 35 g calcium/kg. In room 1 the temperature was maintained at 22°C for 4 weeks, then increased to 32°C for 4 weeks between weeks 24 and 28 and returned to 22°C for 12 weeks. In room 2 the temperature was maintained at 22°C for 12 weeks. It was then increased to 32°C for 4 weeks between weeks 32 and 36 and returned to 22°C for 4 weeks. The relative humidity, actively controlled, was kept constant at 75%. During this period of 20 weeks, egg number and egg weight were recorded daily. Food intake was measured weekly and body weight was measured every 4 weeks.

Analyses

Individual egg production, food intake and body weight data were compared on a 4-week basis using a multifactorial analysis of variance (Anova) and *t* test from Genstat 5 (Lawes Agricultural Trust). For each studied parameter, separate statistical analysis was carried out for each 4-week period.

RESULTS

Experiment 1

Egg production data are presented in Table 1. Hens in the control group supplemented with 10 mg vitamin E/kg showed a significant ($P<0·05$) decline in egg production during heat stress. However, there was no effect of heat stress on egg number in the group supplemented with 500 mg vitamin E/kg. Mean egg weight was depressed in both control ($P<0·005$) and vitamin E-treated ($P<0·05$) groups, but recovered more quickly in the vitamin E-treated group.

Plasma concentrations of calcium, zinc and triglyceride are shown in the Figure. As vitamin E affected none of these characteristics before the stress, the concentrations observed during and after heat stress for a given characteristic were compared to the mean of the pooled pre-heat stress concentrations. Concentrations of calcium, zinc and triglyceride significantly declined in the control

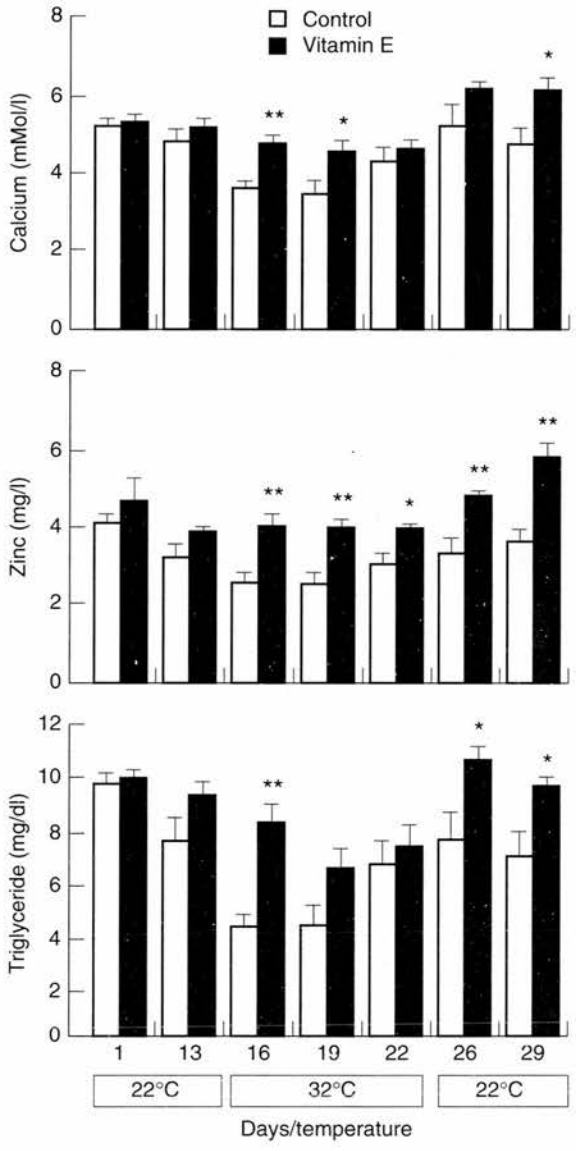

Figure. *Plasma concentrations of calcium (mMol/l), zinc (mg/l) and triglyceride (mg/dl) in control and vitamin-E treated birds. Vertical bars represent SE (Experiment 1). * Significant difference ($P<0·05$) between the treatment for a given sampling time. ** Significant difference ($P<0·01$) between the treatment for a given sampling time.*

Table 1. *Egg production of heat stressed laying hens fed on vitamin E—supplemented diets (10 or 500 mg of α-tocopherol/kg of diet, 12 birds/group) (experiment 1)*

	Days of experiment *			
	1 to 8	8 to 15	15 to 22	22 to 29
Temperature (°C)	22	22	32	22
Egg production (%)				
10 mg/kg vit E	79·2	61·9	51·2[a]	48·8[a]
500 mg/kg vit E	75·0	67·8	72·6[b]	71·4[b]
Significance	NS	NS	$P<0·05$	$P<0·005$
Mean egg weight (g)				
10 mg/kg vit E	65·3[a]	66·0[a]	63·1[a]	63·6[a]
500 mg/kg vit E	67·4[b]	68·7[b]	66·6[b]	67·6[b]
Significance	$P<0·05$	$P<0·005$	$P<0·005$	$P<0·005$

* Within a column, means of a given parameter followed by different letters are statistically different. NS Not significant ($P>0·05$).

group during heat stress: −25% ($P<0·01$), −30% ($P<0·05$) and −45% ($P<0·01$), respectively. Declines were reduced and not significant in the vitamin E-treated group: −10%, 0%, and −19%, respectively. As a result, the concentrations of calcium, zinc and triglyceride were significantly greatest during heat stress in the vitamin E-supplemented group ($P<0·05$, $P<0·01$ and $P<0·05$, respectively) compared to the non-supplemented group. After heat stress, the concentrations of these recovered in both groups but remained significantly higher in the supplemented group ($P<0·05$, $P<0·01$ and $P<0·05$, respectively). In the supplemented group, the concentrations of calcium and zinc rose to become significantly higher than they were before the stress ($P<0·02$ and $P<0·05$, respectively).

Experiment 2

In both rooms, egg production was reduced significantly during the period of heat exposure (−18% in room 1 and −29% in room 2) compared with the previous 4-week period. As a few birds, equally distributed between the treatments (4, 3 and 3 for the groups fed on the diets containing 10, 125 and 500 mg vitamin E/kg, respectively), died during and after heat stress, the number of residual degrees of freedom decreased from 196 at the beginning to 186 at the end of the experiment. Furthermore, as some birds also stopped laying (2, 3 and 0 for the same respective diets), the number of residual degrees of freedom was reduced by a greater extent for mean egg weight and food conversion efficiency (from 196 to 181).

Room 1

The results obtained in room 1 are presented in Table 2. As was observed in experiment 1, dietary vitamin E supplementation did not affect the egg production of birds which had not been exposed to a heat stress (first 4 weeks). During heat stress, the decline in egg production was less for the birds given diets supplemented with high concentrations of vitamin E, especially the group receiving the 500 mg α-tocopherol/kg diet which had a rate of egg production 9·2% higher ($P<0·05$) during the period of heat stress than the control group (10 mg α-tocopherol/kg). During the post-stress period, the higher rate of lay in birds receiving the diet supplemented with high concentrations of vitamin E was maintained for 2 months with an average increase of 10·5% ($P<0·01$) compared to the control group. The group fed on the diet supplemented with 125 mg α-tocopherol/kg showed an intermediate rate of egg production that did not differ significantly from that of the controls.

The average egg weight and food intake declined during the exposure to heat stress but no significant differences were apparent between the vitamin E-supplemented and control groups. As a result, the total egg mass was increased for the birds receiving the highly supplemented diets. It varied from 30·7 g/hen/day for the control group to 35·1 g/hen/day for the group fed the 500 mg α-tocopherol/kg diet during the hot period; however, this difference just failed to reach statistical significance ($P<0·07$). During the 2 months post-stress, the total egg masses for the same respective groups were 32·5 and 37·6 g/hen/day ($P<0·05$). Food conversion efficiency (total egg mass: food consumption, FCE) was improved, though the difference was not statistically significant ($P<0·06$), during the stress period in the group fed the 500 mg α-tocopherol/kg diet compared to the control group. This difference in FCE was maintained during the 2 month period following heat stress.

Room 2

The results obtained in room 2 are presented in Table 3. Although the groups fed on the vitamin E-supplemented diets produced more eggs during and after the stress period than the control group, the differences failed to reach significance ($P<0·1$ during the stress period and $P<0·07$ after the stress). As at the end of the first 4 weeks, the birds in this room were slightly heavier (1,592 g, average for the 3 treatments) than in room 1 (1,543 g); total egg mass and food intake were also slightly higher in room 2 than in room 1 but again there were no significant treatment effects on mean egg weight, total egg mass, food intake or FCE.

DISCUSSION

The results of these experiments show that supplementing diets of laying hens with a relatively high concentration of α-tocopherol (500 mg/kg) can reduce the detrimental effect of chronic heat stress upon egg production. The greatest effect was achieved in experiment 1 where egg production was 22% higher in the supplemented group during the week of heat stress compared with the controls. In the second experiment, the average benefit during the 4-week stress period was 7% (9% and 5% in rooms 1 and 2, respectively). However, the overall benefits to egg production in both experiments extended beyond these periods of stress, because egg production of hens given a high concentration of vitamin E recovered faster after the removal of the heat stress. Effects of vitamin E on egg weight in stressed hens were inconsistent: a beneficial response was seen in experiment 1 but there was no effect in experiment 2. The difference in response is unlikely to be related to the age of the hens, because hens in experiment 1 were stressed at about the same age as those in room 2 in experiment 2. Instead, the difference may be related to the duration of the stress (4 compared with 1 week) or to egg weight. Indeed, egg weight

Table 2. *Egg production of laying hens fed on vitamin E-supplemented diets (10, 125 or 500 mg α-tocopherol/kg of diet, 50 birds/group) in room 1 and subjected to a period of chronic heat stress between 24 and 28 weeks of age (experiment 2)*

	Periods of experiment (weeks of age)*				
	20 to 24	24 to 28	28 to 32	32 to 36	36 to 40
Temperature(°C)	22	32	22	22	22
Egg production (%)					
10 mg/kg vit E	74·2	56·2^{a}	55·2^{a}	55·0^{a}	66·6^{ab}
125 mg/kg vit E	74·2	61·1^{a}	55·4^{a}	56·3^{a}	62·8^{b}
500 mg/kg vit E	76·2	65·4^{b}	64·4^{b}	66·7^{b}	72·6^{a}
Significance	NS	$P<0·05$	$P<0·01$	$P<0·01$	$P<0·01$
SED	3·8	4·2	3·8	4·0	3·5
DF (residual)	196	194	194	188	186
Mean egg weight (g)					
10 mg/kg vit E	52·2^{a}	54·4	56·6	59·4	62·4^{a}
125 mg/kg vit E	50·7^{b}	53·0	54·7	58·3	60·6^{b}
500 mg/kg vit E	51·1^{ab}	53·6	55·7	58·7	60·6^{b}
Significance	$P<0·05$	NS	NS	NS	$P<0·05$
SED	0·6	0·7	0·8	0·9	0·8
DF (residual)	195	194	193	187	181
Food intake (g/d)					
10 mg/kg vit E	79·5	60·3	77·6	85·9	94·8
125 mg/kg vit E	78·2	60·0	74·6	79·8	87·9
500 mg/kg vit E	80·0	62·4	80·8	85·4	94·2
Significance	NS	NS	NS	NS	NS
SED	3·3	3·6	3·5	3·7	3·7
DF (residual)	196	194	194	188	186
FCE ** (g/g)					
10 mg/kg vit E	0·43	0·44^{ab}	0·34	0·23^{a}	0·45
125 mg/kg vit E	0·46	0·43^{a}	0·30	0·32^{ab}	0·33
500 mg/kg vit E	0·47	0·53^{b}	0·43	0·43^{b}	0·46
Significance	NS	$P<0·05$	NS	$P<0·05$	NS
SED	0·08	0·04	0·06	0·05	0·14
DF (residual)	195	194	193	187	181

* Within a column, means of a given variable followed by different letters are statistically different. NS Not significant ($P>0·05$).
** FCE Food conversion efficiency (g of total egg mass/g of food).

was considerably higher before the stress in experiment 1 (66 g) than in experiment 2 (52 and 58 g in rooms 1 and 2, respectively), even though the hens were from the same strain.

In experiment 2, total egg mass over the stress period was significantly higher in hens given the diet with the highest dietary vitamin E concentration. Food intake was also higher than in the control group with this treatment, though the difference was not statistically significant. The overall effect was an improvement in FCE with the vitamin E treatment, which represents an additional commercial advantage to the use of vitamin E under conditions of heat stress.

Adverse metabolic effects of heat stress that were observed included decreased plasma concentrations of total calcium and the egg yolk precursors, vitellogenin and triglyceride. Heat stress has previously been shown to depress plasma total calcium (Wolfenson *et al.*, 1979) and blood ionised calcium (Odom *et al.*, 1986). The maintenance of more normal plasma concentrations of vitellogenin (zinc), VLDL (triglyceride) and calcium by dietary supplementation with 500 mg of α-tocopherol/kg diet suggests that the decreased production of egg precursors is an important factor in the decline in egg production seen during heat stress and that the beneficial effect of vitamin E on egg production acts in a manner which prevents the decline of these precursors in the circulation.

The reductions in circulating vitellogenin, triglyceride and calcium in heat stressed hens may be indicative of cellular dysfunction associated with impaired membrane structure. Exposure to heat stress may lead to an excessive production of oxygen free radicals which damage cell membranes by peroxidation of PUFAs. In an *in vitro* muscle preparation, changes in membrane integrity induced by an ionophore to simulate the effects of heat stress resulted in a release of intracellular enzymes (for example, creatine kinase) and accumulation of intracellular calcium. Both of those changes were shown to be inhibited by vitamin E (Mitchell *et al.*, 1994). Likewise, in rats treated with dehydroepiandrosterone to induce hepatic oxidative injury, vitamin E prevented the associated rise in specific antioxidant enzymes and markers of cellular damage (McIntosh *et al.*, 1993). These observations confirm an important role for the vitamin in maintaining cell membrane integrity under stressful conditions.

Loss of membrane integrity can be implicated in a reduction in the formation of ATP and decline in cellular metabolism during stress. This damage can be particularly serious in organs such as muscle and liver because of their high metabolic activity

Table 3. *Egg production of laying hens fed on vitamin E-supplemented diets (10, 125 or 500 mg α-tocopherol/kg of diet, 50 birds/group) in room 2 and subjected to a period of chronic heat stress between 32 and 36 weeks of age (experiment 2)*

	Periods of experiment (weeks of age)*				
	20 to 24	24 to 28	28 to 32	32 to 36	36 to 40
Temperature (°C)	22	22	22	32	22
Egg production (%)					
10 mg/kg vit E	77·6	82·0	74·0	44·7	47·8
125 mg/kg vit E	75·4	83·0	78·6	48·1	52·1
500 mg/kg vit E	78·7	80·5	74·5	49·9	53·9
Significance	NS	NS	NS	NS	NS
SED	3·8	4·2	3·8	4·0	3·5
DF (residual)	196	194	194	188	186
Mean egg weight (g)					
10 mg/kg vit E	52·7[a]	56·2	58·1	57·4	59·7
125 mg/kg vit E	51·0[b]	55·3	57·6	56·9	59·4
500 mg/kg vit E	51·0[b]	55·2	57·8	57·4	59·3
Significance	$P<0·05$	NS	NS	NS	NS
SED	0·6	0·7	0·8	0·9	0·8
DF (residual)	195	194	193	187	181
Food intake (g/d)					
10 mg/kg vit E	85·5	89·8	84·6	55·5	77·7
125 mg/kg vit E	82·7	92·7	89·4	57·0	80·4
500 mg/kg vit E	82·7	89·5	86·0	60·6	80·8
Significance	NS	NS	NS	NS	NS
SED	3·3	3·6	3·5	3·7	3·7
DF (residual)	196	194	194	188	186
FCE ** (g/g)					
10 mg/kg vit E	0·45	0·49	0·50	0·39	0·25
125 mg/kg vit E	0·34	0·47	0·40	0·44	0·34
500 mg/kg vit E	0·46	0·47	0·50	0·42	0·37
Significance	NS	NS	NS	NS	NS
SED	0·08	0·04	0·06	0·05	0·14
DF (residual)	195	194	193	187	181

* Within a column, means of a given variable affected by different letters are statistically different.
NS Not significant ($P>0·05$).
** FCE Food conversion efficiency (g of total egg mass/g of food).

(Fowler, 1990). Increased membrane lipid peroxidation and heat stress-induced liver dysfunction may thus contribute to the decreased plasma concentrations or reduced hepatic synthesis of vitellogenin and triglyceride observed in heat-stressed laying hens given the control (10 mg vitamin E/kg) diet in the present study. The dietary supplementation with 500 mg α-tocopherol/kg may therefore have afforded a degree of protection to the liver during heat stress and thus increased the availability of egg yolk precursors for egg formation by reducing oxidative stress.

Another possible explanation for the metabolic changes would involve a direct effect of oestradiol. Hepatic synthesis of vitellogenin and triglyceride is under direct oestrogenic control. Likewise, oestradiol has an effect on circulating calcium through its control of the synthesis of 1,25-dihydroxycholecalciferol (Taylor and Drake, 1984), the active cholecalciferol metabolite that regulates calcium absorption. Circulating calcium and oestrogen concentrations are highly correlated in laying hens (Tojo and Huston, 1980) and oestradiol concentrations have been shown to be depressed in hens subjected to heat stress (Tojo and Huston, 1980; Mahmoud *et al.*, 1995). Vitamin E might influence these oestradiol-dependent mechanisms by exerting a direct effect on oestradiol or an indirect effect through maintaining more normal function of cellular processes regulating oestradiol. These 2 mechanisms, protection of the liver or other organs against oxidative damage and restoration of oestrogen secretion or sensitivity could, independently or together, explain the effects of high concentrations of vitamin E on the egg yolk precursor economy and improved egg production in heat-stressed laying hens.

These experiments, therefore, showed that 500 mg vitamin E/kg reduced significantly the detrimental effect of chronic heat stress upon egg production, total egg mass and FCE in laying hens. The beneficial effect of vitamin E during and after heat stress was also apparent on the circulating amounts of egg yolk precursors (vitellogenin, VLDL and calcium). This may either have been caused by a protective effect of vitamin E on the membrane of hepatocytes or by the effect of vitamin E on oestradiol concentration or activity. Further trials will therefore investigate the influences of vitamin E on vitellogenin, oestradiol and liver lipid peroxidation during heat stress and will determine the optimal dose and duration of dietary vitamin E supplementation required to alleviate the effect of heat stress in laying hens under

commercial conditions. Through a better understanding of the mechanisms controlling egg production, including those involved in regulating the synthesis, transport and oocyte incorporation of egg yolk precursors and the actions of vitamin E upon these processes, it should be possible to formulate more effective nutritional strategies to alleviate or minimise the detrimental effects of chronic heat stress upon laying hens.

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